

which had been desiccated in various ways. From these figures it is clear that although removal of water by means of acetone did not result in any obvious loss of nitrogen-retaining activity, desiccation of the gland with alcohol under conditions similar to those advocated by Collip [1939] resulted in an extract almost completely devoid of such activity.

Animals receiving protein and carbohydrate moieties of the diet at different times

The results of two typical experiments are illustrated graphically in Figs. 1 and 2. In both instances the daily injection of a crude alkaline extract of fresh ox anterior lobe was followed by a sharp fall in the daily nitrogen excretion, which was maintained as long as the daily injections were continued. Cessation of treatment was followed by a sudden rise of nitrogen excretion to a level above that in the pre-injection control period.

It was to be expected from previous results [Cuthbertson *et al.*, 1940] that the separation in time of dietary carbohydrate and protein under these conditions would result in a negative nitrogen balance, while giving the carbohydrate and protein simultaneously should result in approximate nitrogen equilibrium. The data in Table III, which refers to the

Table III. *Influence of crude alkaline extract of fresh ox anterior lobe on nitrogen balance of rats receiving dietary carbohydrate and protein at different times*

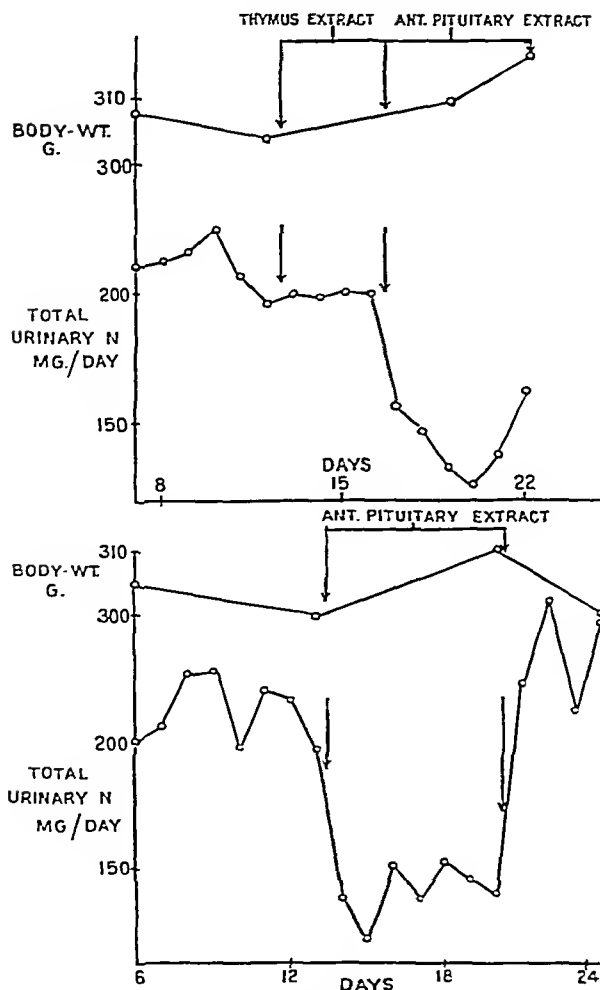
Experiment number	Average daily faecal nitrogen excretion (mg./rat)	Average daily urinary excretion of nitrogen (mg./rat)			Average daily intake of nitrogen (mg./rat)	Nitrogen balance (mg./rat/day)		
		Pre-injection period	Injection period	Post-injection period		Pre-injection period	Injection period	Post-injection period
1	—	211	145	—	225	-24*	+42*	—
2	38	212	142	236	225	-25	+45	-49

* Based on the assumption that faecal nitrogen excretion was constant at 38 mg./rat/day.

experiments the results of which are illustrated in Fig. 1, show that a negative nitrogen balance was indeed found during the pre-injection control period, but that a substantial nitrogen storage was induced by the treatment with pituitary extract. When daily treatment with the extract was stopped the nitrogen balance became substantially more negative than it had been during the pre-injection control period.

DISCUSSION

Harrison & Long [1940] demonstrated that the administration of a crude anterior pituitary extract to fasting rats results in a marked reduction in the urinary excretion of nitrogen, and our results are in agreement with theirs, in so far as rats receiving a constant and limited amount of mixed diet are concerned.



FIGS. 1 & 2

FIGS. 1 and 2. Influence of daily treatment with anterior pituitary extract on the body-weight and on the increased protein catabolism which follows separation in time of the protein and carbohydrate moieties of the diet of the adult rat. The figures plotted are the averages for groups of rats.

Extract of thymus gland or of anterior pituitary gland was administered subcutaneously each day during the periods indicated by the arrows.

It is interesting that desiccation of ox anterior pituitary tissue with alcohol, as advocated by Collip [1939] results either in an inactivation of the substance responsible for the nitrogen-retaining action of pituitary extracts, or in such alteration of its properties that it becomes insoluble.

under the conditions which permit its extraction from fresh tissue or from acetone-desiccated material. The use of acetone-desiccated tissue or of the fresh gland itself seems preferable for the production of simple active extracts.

Nevertheless, the use of extracts prepared from alcohol-desiccated anterior lobe tissue may assist in the differentiation of the nitrogen-retaining substance from certain other pituitary hormones, such as prolactin, which are present in a crude alkaline extract of the alcohol-desiccated material. It may be mentioned that an aqueous alcoholic extract of fresh or of acetone-desiccated ox pituitary tissue was not found to possess nitrogen-retaining activity under the condition of our experiments, nor was activity found in an alkaline extract of the commercial acetone-desiccated ox pituitary powder which had previously been found to be deficient in certain other pituitary hormones [Chance, Rowlands & Young, 1939]. It is clear, therefore, that the active principle is somewhat more labile than some other pituitary factors, a fact in keeping with the observations of Harrison & Long [1940]. In subsequent investigations a crude alkaline extract of fresh anterior lobe tissue was employed as a source of the pituitary nitrogen-retaining substance.

It was expected that an increased rate of body-growth would be found when rats were treated with these crude pituitary extracts and such was found to be the case (Table I). It is interesting, but perhaps fortuitous, that the dose-growth response curve for crude ox pituitary extract implicit in the results in Table I is very similar to that previously published by Marks & Young [1940] who used adult female rats for the assay of growth hormone, according to the method of Evans & Simpson [1931]. If this similarity is more than a coincidence it suggests that the male rat, when its growth curve has been 'plateaued' by restriction of diet is capable of responding for a short time to the growth-stimulus of the anterior pituitary extract, to the same extent as the older female rat, whose growth curve has plateaued naturally despite unlimited food intake. In both instances, therefore, the limitation to growth may be the limitation of ability to store the nitrogen absorbed in the food as body protein in a form which is not a labile one.

In both series of experiments the period of nitrogen retention induced by treatment with the pituitary extract was followed by a period of increased nitrogen loss, as though some part of the nitrogen had been retained in a loosely bound form. In the experiments with the mixed diet, this post-injection period of increased protein catabolism was not associated with any obvious or general loss of body-weight, although growth was slight or non-existent during this period. In the experiments in which the heavier rats received their dietary carbohydrate and protein

at different times, the animals lost body-weight during the post-injection period when the excessive loss of nitrogen occurred.

It is clear from our results that the nitrogen loss associated with the separation in time of the carbohydrate and protein portions of the diet of the adult rat, can be more than overcome by treatment with anterior pituitary extract, but this does not necessarily mean that the pituitary gland controls this particular relationship between the metabolism of carbohydrate and that of protein. Experiments on hypophysectomized rats would be required to settle this point.

SUMMARY

1. Although crude extracts of fresh ox or sheep anterior pituitary gland, and of acetone desiccated ox anterior pituitary tissue, are capable of inducing nitrogen retention in rats with a restricted daily intake of a mixed diet, extracts prepared from ox anterior pituitary tissue which has been desiccated in alcohol, are almost completely inactive in this respect.

2. The increased nitrogen loss associated with the separation in time of the carbohydrate and protein moieties of the diet of the adult rat can be more than compensated by the retention of nitrogen induced by treatment with a crude extract of fresh ox anterior pituitary tissue.

3. In all these experiments, the period of increased nitrogen retention under the influence of pituitary extract was followed by a period of excessive nitrogen loss.

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THE ANTERIOR PITUITARY GLAND AND PROTEIN METABOLISM

II. THE INFLUENCE OF ANTERIOR PITUITARY EXTRACT ON THE METABOLIC RESPONSE OF THE RAT TO INJURY

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(Received 15 May 1941)

BOTH in the human being and in the rat an injury, such as the fracture of a long bone, causes an increased output of nitrogen and creatine in the urine. The excretion of sulphur, phosphorus and potassium is also increased, indicating a breakdown of tissue substance. This was found to be a general, rather than a local, breakdown of tissue substance [Cuthbertson, McGirr & Robertson, 1939].

In the preceding paper [Cuthbertson, Webster & Young, 1941], the ability of a crude extract of anterior pituitary tissue to induce nitrogen retention was confirmed, and it was shown that the loss of nitrogen associated with the separation in time of the carbohydrate and protein constituents of the diet of the adult rat could be more than overcome by the injection of a crude extract of the anterior pituitary glands of oxen. It was therefore of some interest to investigate the influence of such an extract on the excessive protein catabolism of trauma in the rat, and on the rate at which recovery from the effects of the injury occurred.

METHODS

Animals and diet

Adult Wistar-strain, male or female albino rats were used in this investigation. They received a constant amount of food each day (Rowett Institute stock diet [Thomson, 1936]), of such amount that they either remained at a relatively constant weight, or slightly lost in weight. This slight restriction in normal food intake is necessary to ensure that the animals will still eat their full ration after the operation. If the consumption of food falls after the operation the interpretation of the results is rendered doubtful.

Procedure

In all 14 operated animals were treated with anterior pituitary extract, while 15 similarly operated control rats were also examined. Three

successive experiments were carried out in which the animals were distributed as follows:

Experiment 1: 4 untreated control male rats

4 pituitary-treated male rats.

Experiment 2: 5 thymus-treated control male rats

5 pituitary-treated male rats.

Experiment 3: 6 thymus-treated control female rats

5 pituitary-treated female rats.

The pituitary-treated rats received daily subcutaneous injections of 1 ml. of a crude alkaline extract of fresh ox anterior pituitary tissue, equivalent to 50 mg. of dried tissue. In two experiments the control animals were treated similarly with an extract prepared in a comparable manner from calf thymus gland.

The general procedure for the collection of urine and for the determination of nitrogen balance was similar to that in previous investigations [Cuthbertson *et al.*, 1939]. Urine collection from metabolism cages was carried out during the last 5 days of an 8-day pre-operation period on the stock diet. At the end of this period the rats were anaesthetized with open ether and incised over a depilated area on the left thigh. A fracture of the left femur was then produced by crushing with forceps, after separation of the muscle groups down to bone by blunt dissection had been effected. The wound was then closed and allowed to heal aseptically. The daily injection of pituitary or thymus extract was begun on the day of operation and continued for 8 days after.

At the end of the experiment the animals were killed with chloroform, and after the carcasses had been skinned, the hind limbs were cut into symmetrical halves by first truncating the body above the level of the iliac crests at the first and second lumbar vertebrae and then splitting the symphysis pubis and sacral regions longitudinally. The limbs were then weighed. The quadriceps femoris, and in some cases also the gastrocnemius, of each limb was then removed and weighed, although this was rendered somewhat difficult in the injured limbs by the strong adhesions of the quadriceps around the fracture.

RESULTS

Nitrogen metabolism during trauma due to injury

Total nitrogen excretion

Untreated control animals. The production of a fracture of the femur produced a sharp rise in the excretion of urinary nitrogen (Fig. 1) which reached a maximum value on the third day after injury. The subsequent fall in nitrogen excretion was also sharp, and basal conditions were

reached in approximately one week after injury. The results were closely parallel to those found under similar conditions in an earlier experiment, as indicated by the broken line in Fig. 1 [Cuthbertson *et al.*, 1939].

Thymus-treated control animals. The two experiments, the average values of which are shown in Fig. 2, differed somewhat in their results. One showed a sharp rise in nitrogen excretion with a fairly sharp fall

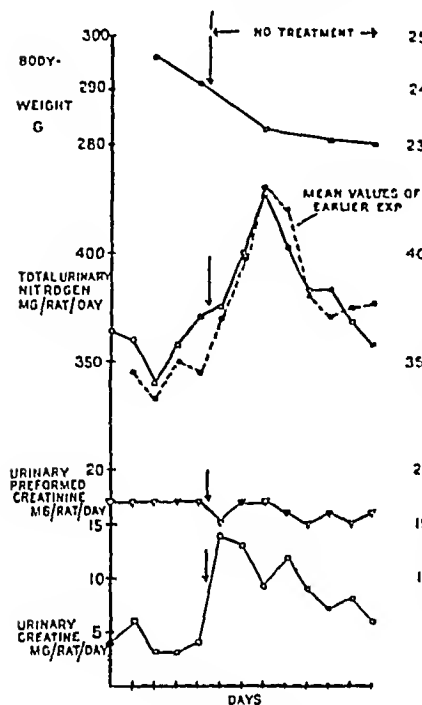


FIG. 1. Untreated control rats.

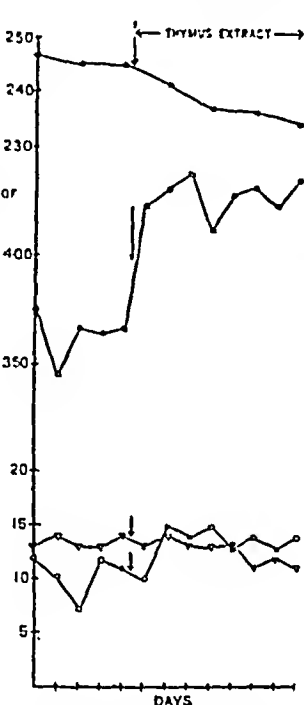


FIG. 2. Control rats receiving daily subcutaneous injections of thymus extract.

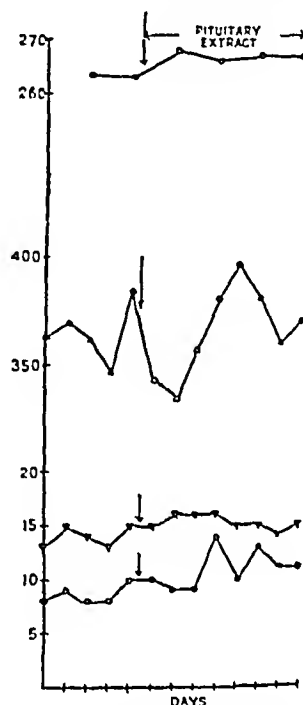


FIG. 3. Rats given daily subcutaneous injections of anterior pituitary extract.

FIGS. 1, 2 and 3. Influence of fracture of the left femur on the body-weight and on protein catabolism in the rat. The arrows indicate the day on which the fracture was induced. The figures plotted are average values for the experiments.

during the next two days, although thereafter the level tended to remain high. In the other experiment (female animals) the rise in urinary nitrogen was slow and reached its maximum only on the sixth day. This slow rise in the second group of thymus-treated control rats was also reflected in the creatine excretion, which tended to parallel the changes in nitrogen elimination. This slow reaction is unusual and we can offer no explanation for this result in one experiment.

When the results for these two groups are averaged the mean values (Fig. 2) indicate a sharp rise in nitrogen excretion, with a maximum value on the third day, after which the level tends to remain high.

Anterior-pituitary treated animals. In all three of the experiments the results of which are summarized in Fig. 3, there was a marked fall in

nitrogen excretion during the two days following injury. Thereafter the urinary nitrogen output tended to rise, reaching a maximum value on the fifth day. Over all there was no loss during the first five post-injury days and no significant loss over eight days (Table I). The period of five days was selected in order to conform with the number of pre-injury observations.

Table I. *Mean daily urinary nitrogen excretion in mg./rat over two five-day periods. Figures in parenthesis are values for eight-day periods*

Treatment	Pre-injury	Post-injury	N balance
Untreated controls	359	397 (387)	-38 (-28)
Thymus extract	363	427 (425)	-64 (-62)
Anterior pituitary extract	365	364 (366)	+1 (-1)

Creatine excretion

In Table II are the mean values for the five pre-injury and five post-injury days. Figures in parenthesis refer to eight-day periods. The rise in creatine excretion tended on the whole to occur earlier than that in the excretion of nitrogen, but the small rise for the thymus-treated, in comparison with that for the untreated, rats is surprising.

Table II. *Mean daily creatine excretion in mg./rat*

Treatment	Pre-injury	Post-injury	Balance
Untreated controls	4	11 (10)	-7 (-6)
Thymus extract	10	13 (14)	-3 (-4)
Pituitary extract	9	10 (11)	-1 (-2)

The excretion of preformed creatinine remained remarkably constant in spite of the variations in creatine output (Figs. 1, 2, & 3) a finding which we have previously noted, and which would suggest that the normal mechanism for the conversion of creatine to creatinine is not functioning effectively.

Response of muscles to injury

Weight of hind limbs

Owing to the difficulty in effecting a truly symmetrical bi-section the values in Table III are to be regarded as very approximate. It is to be noted, however, that not only was the difference between normal and injured limb greatest in the anterior-pituitary-treated group, but that even the injured limbs in this group were of higher weight than the corresponding limbs of the other groups. On the whole the increase in weight of the normal limbs is in proportion to the general increase in body-weight in the pituitary group.

Table III. *Limb and muscle weights in g. (Left limb damaged)*

	Hind limbs			Quadriceps fem.			Gastrocnemius		
	Right	Left	Diff.	Right	Left	Diff.	Right	Left	Diff.
Anterior-pituitary-treated rats	26.75	25.06	-1.69	2.372	2.115	-0.257	1.476	1.326	-0.150
Thymus-treated rats	22.64	21.49	-1.15	2.024	1.754	-0.270	1.113	1.006	-0.107
Untreated control rats	23.66	24.07	+0.41	2.357	2.150	-0.207	1.401	1.372	-0.029

It should be made clear that the anomalous finding of no loss in limb weight in the group of untreated controls is contrary to previous observations and emphasizes the general unreliability of this particular measurement.

Quadriceps femoris

It is obvious that the loss of nitrogen in the urine of the control rats cannot be explained by differences in the weights of the hind limbs or quadriceps femoris (Table III). This is in agreement with earlier findings. Such slight increase in weight of the quadriceps muscles of both sides in the anterior pituitary treated group was a reflection of the change in total body-weight.

The difference between right and left legs showed little change from group to group. Such differences as occurred were probably conditioned by the nature of the injury, which, although initially produced in approximately the same way, yet yielded different results as regards fragmentation of bone and degree of over-riding, etc.

Gastrocnemius

This muscle also shared in the general difference between normal and injured legs but to a less marked extent (Table III).

Moisture content of muscles

Such differences as exist between the total weights of these muscles, of the anterior-pituitary-treated group and the thymus-treated animals were not due to a rise in water-content of the muscles of the former.

Total body-weights

Following trauma, and during the period of injection, the anterior pituitary group gained slightly in weight (Fig. 3). The increment occurred during the early days and was maintained. The other groups showed a fall in body-weight of some 10.5 g., equivalent to about 329 mg. of nitrogen, if we are justified in considering the loss of weight to be entirely due to wasting of muscle. The actual loss of nitrogen by the untreated controls and the thymus-treated controls averaged 360 mg. over the 8 days. Considering the gross assumptions the discrepancy is probably not significant.

DISCUSSION

The daily administration of a crude alkaline extract of anterior pituitary gland to rats suffering from a fractured femur produced by open operation prevented the loss in body-weight and excessive loss of nitrogen and creatine, which are the usual concomitants of such an injury. Injections of thymus extract caused a rather greater loss of nitrogen than would have occurred if the animals had been untreated, but this was most probably due to the manipulation of the injured animal during the injection process, which resulted in a disturbance of the zone of fracture. We have some evidence that, in the human organism, resetting a fracture may cause a disturbance of metabolism. That the thymus extract *per se* would not be expected to induce a rise in nitrogen excretion, is seen from the results of the preceding publication [Cuthbertson, Webster & Young, 1941]. In view of this it is all the more remarkable that the injection of the anterior pituitary extract, which required a similar manipulation, should have led to little or no loss of nitrogen.

The effect of the pituitary extract, like that of the injury, on the metabolism of the injured animal, is a general rather than a local effect, and possibly represents the normal growth response to this extract, superimposed upon and compensating the increased catabolism following injury.

Although there was *a priori* no ground for believing that desoxycorticosterone should cause nitrogen retention during the post-injury period, since its general effects are otherwise, nevertheless we thought the point worth investigation. The dose used for intramuscular injection was 0.5 mg. desoxycorticosterone acetate (Ciba 'Percorten') per day. The 5 fractured animals in this group showed a rapid rise in nitrogen excretion which also reached the maximum value on the third day. The rise was more sustained than in the untreated controls and conformed to the male group of thymus-treated control rats. The creatine excretion reached its maximum on the first and second days and then declined slowly.

The observations made on the rate at which the atrophied muscles are restored under the influence of the pituitary extract show that although the animal as a whole grows more rapidly under the pituitary stimulus, and protein catabolism in general is diminished, no greater rate of restitution of the atrophied muscles of the injured limb is thus induced. In other words, the promotion of protein anabolism in general by the pituitary extract provided no particular stimulus for the restoration of the protein which had been lost, as the result of trauma, by the muscles of the injured limb. The extract is therefore of no obvious use in the clinical treatment of such muscle atrophy.

Nevertheless the possibility remained that the extract might be of value in inducing more rapid restoration of tissue which had been surgically removed, i.e. that it might be of use in promoting the restoration of tissue lost as a result of wounds. An investigation of this point forms the subject of the following paper [Cuthbertson, Shaw & Young, 1941].

SUMMARY

The daily administration of a crude alkaline extract of ox anterior pituitary lobe to rats suffering from a fracture of one femur prevents the loss of body-weight and excessive excretion of nitrogen and of creatine which is the normal result of such an injury. No beneficial treatment was observed on the restoration of the atrophied muscles of the injured limb.

We should like to thank Prof. E. P. Cathcart and Prof. G. M. Wishart for their stimulating interest in this work, and to express our thanks to the Medical Research Council and to the Rankin Medical Research Fund for their financial support. We are much indebted to Ciba Ltd. for a supply of 'Percorten'.

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THE ANTERIOR PITUITARY GLAND AND PROTEIN METABOLISM

III. THE INFLUENCE OF ANTERIOR PITUITARY EXTRACT ON THE RATE OF WOUND HEALING

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IN the preceding paper [Cuthbertson, Shaw & Young, 1941] we showed that treatment with a crude alkaline extract of ox anterior pituitary lobe can prevent the loss of body-weight and the excessive loss of urinary nitrogen and creatine which normally follow fracture of the femur in the rat, although such treatment did not increase the rate of restoration of the muscles which atrophied as the result of the injury. At the request of the Medical Research Council we have examined the effect of a similar extract on the rate of wound healing in the rat.

Arey [1936] who has summarized the literature on wound healing finds little or no work relating to the action of the anterior pituitary gland on the rate at which wounds heal; apart from the observations of Ceccarelli [1930] who noted that local applications of an extract had no influence on the total healing period, and Lauber [1933] who obtained favourable results with injections of 'hypophysin' and 'Prolan'.

METHODS

Animals and diet, &c.

Sixty-eight Wistar-strain male albino rats of average weight 215 g. were used in the present investigation. They were given the stock diet [Thomson, 1936] *ad libitum* throughout the experiment.

After the experimental production of the wounds, 34 of the animals received daily subcutaneous injections of 1 ml. of a crude alkaline extract of fresh anterior lobe tissue of ox pituitary gland, each ml. being equivalent to 50 mg. of dried tissue. The injections were begun on the day of operation and continued until the wounds were completely healed. The control animals remained untreated.

Production of wounds and measurement of rate of wound healing

The animals were anaesthetized with open ether and depilated over the outer aspect of each thigh by plucking. Two circular incisions were

demarcated, one on each side, by a specially sharpened cork borer of 12.8 mm. diameter. The circular incisions so produced did not completely pierce the skin as the under-lying muscle is very easily injured. The areas thus marked out were then carefully cut out with scissors, and the circular discs of skin released from the underlying muscle by cutting through the adherent connective tissue. The wounds subsequently enlarged but remained roughly circular in outline.

The first measurements were made 8 hours after the injury, and then at daily intervals. After 24 hours the wound was rather smaller than its original size, the diminution mainly affecting the diameter corresponding to the long axis of the thigh. The wounds therefore tended to heal as ellipses in which the major axis initially diminished less rapidly than the minor axis—a difference which was maximal during the first two to three days, and then gradually disappeared as the rate of decline of the minor axis gradually fell off with time. Measurement of major and minor axes was effected by means of dividers. Even if the wounds were not true ellipses any error made in calculating the area on this assumption will not affect the general comparison.

About the fifth day after the operation the hard scabs which had previously formed tended to be 'pushed off' the surface of the granulating wound by the epithelization underneath. It was difficult at this stage exactly to determine the true axes of these wounds, but, by laying the divider points underneath the edges of the scab it was possible to obtain a reasonable assessment of the size of the wound. When the scabs fell off, or were rubbed off by the animal, the granulating surface could be more accurately measured. The wound finally ended as a very small narrow line. None of the wounds became septic.

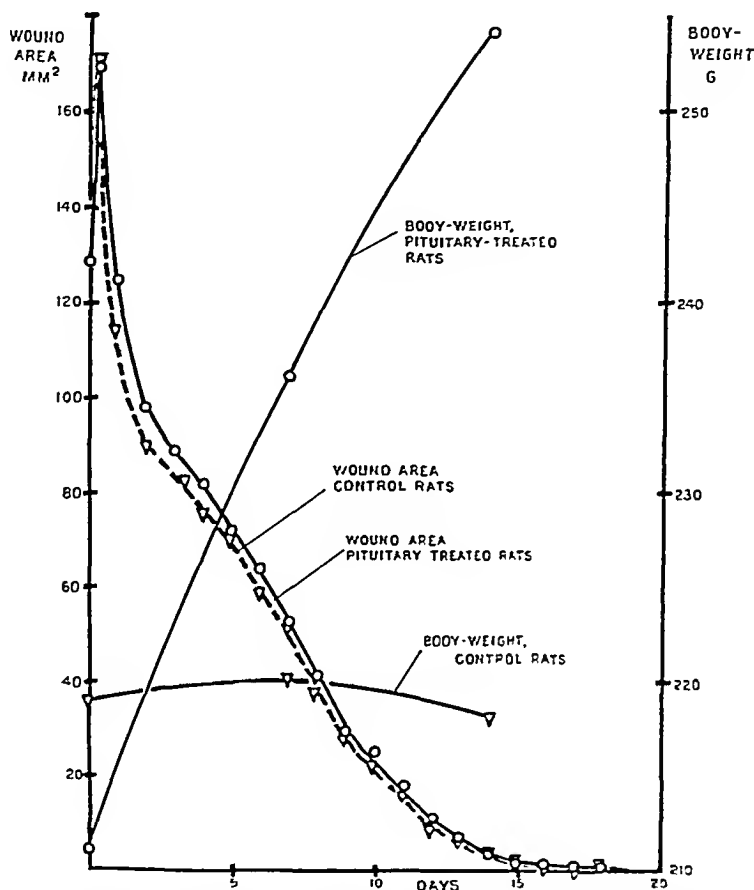
The criteria of complete healing were the disappearance of all granulation tissue, and the complete closure of the wound. Further observation was impossible short of excising the scar and measuring its tensile strength, which was not done.

RESULTS

The results are summarized in the Table which shows that the anterior pituitary extract exerted no accelerating action on the rate at which the wounds healed (see Graph).

In the animals treated with anterior pituitary extract the process of wound shrinkage was slightly less rapid than that in the control rats (Graph), and the pituitary-treated animals therefore commenced the final period of epidermization with slightly larger wounds. How far this was due to the process of injection and how far to the extract is impossible to state, but in any case the difference is very slight and mainly affected the minor axes. The total weighted mean period of healing was essentially

the same for both groups, namely 15.1 days for the control rats and 15.5 days for the anterior pituitary-treated animals.



Influence of daily subcutaneous injections of an extract of anterior pituitary gland on the body-weight and on the rate of wound healing, in the adult male rat.

DISCUSSION

The initial increase in size of the wound was the result of removing a disc from a relatively elastic and mobile skin. In the rapid restoration of the breach, which began within 24 hours, shrinkage or some physico-chemical process appeared to be the main factor. Epithelial migration resulting in epidermization completed the work of contraction [see also Carrel & Hartmann, 1916]. This process varies directly with the size of the wound. The larger the wound the greater the rate of cicatrization (see Graph).

We may safely conclude from our results that the crude ox anterior lobe extract, which was known to prevent excessive traumatic catabolism of tissue protein in the rat [Cuthbertson *et al.*, 1941] exerts no accelerating action on the rate at which these artificially induced wounds heal, although it stimulates general body growth (see Graph).

Influence of crude pituitary extract on rate of wound healing in rats

	Mean size of wound on second day (mm. ²)	Days to complete healing (weighted mean)	Rate of healing (mm. ² /day)
Control rats	89.74	15.1	5.9
Anterior-pituitary-treated rats	97.88	15.5	6.3

SUMMARY

A crude alkaline extract of the anterior pituitary of the ox which was growth-promoting and capable of preventing loss of body-weight and tissue nitrogen in rats with fractured femurs, was found to have no significant effect on the total period required to heal wounds caused by the removal of circular discs (128.7 mm.²) of skin from the upper thighs of rats.

We wish to express our thanks to Professor J. S. Young of the University of Aberdeen for much useful advice on the production of wounds.

This work was carried out with the aid of financial support from the Medical Research Council, to whom we express our thanks.

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USE OF OESTROGENIZED MALE RATS FOR THE STUDY OF GONADOTROPHIC ACTIVITY

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CHRONIC oestrogenization of male animals causes retardation of body growth and atrophy of the testes, both effects being due mainly, if not exclusively, to depression of hypophyseal activity. A review of the literature has recently been given by Deanesly [1939]. Injection of growth hormone and gonadotrophins during the course of oestrogen treatment will maintain growth and prevent the atrophy of the reproductive organs [Spencer, D'Amour & Gustavson, 1932; Moore & Price, 1932].

It seems unlikely that chronic oestrogenization will completely obliterate the somato- and gonado-trophic functions of the anterior pituitary gland, i.e. will lead to the same condition as hypophysectomy, and certain differences in the behaviour of oestrogenized and hypophysectomized rats have been pointed out as regards growth and response to gonadotrophins [Noble, 1939]. Nevertheless, oestrogenized rats are definitely quiescent as regards gonadal activity, and the growth above 120 g. is negligible. Such animals might therefore be used for the assay of somato- and gonadotrophic preparations. Experiments on these lines with growth hormone have been recorded by Levie [1939] and analogous ones with gonadotrophins are described below.

MATERIAL AND TECHNIQUE

Male rats of not less than 100 g. at the beginning of the experiment were used. At this size they are beginning to show full sensitivity to oestrogenization. Oestrogen was administered in the form of diethylstilboestrol, two 15-mg. tablets of which were implanted subcutaneously, except in one experiment, under ether anaesthetic, just before injections began. The amount absorbed during the 20-day experiments averaged about 5 mg. from each tablet. This dosage was probably unnecessarily large for depression of the pituitary activity, but it was thought desirable to give over- rather than under-dosage. With stilboestrol the anti-pituitary action is the same as that of other oestrogens [Noble, 1938; Gaarenstroom & de Jongh, 1939] but rather more intense over short periods owing to its greater subcutaneous solubility [Deanesly & Parkes, in press]. Sections through the testes from 138 rats belonging to 23 experimental

groups were examined to find out what stages of spermatogenesis were present.

EXPERIMENTAL RESULTS

Oestrogenization only

Table I shows the average size of testes and seminal vesicles in normal and oestrogenized adult rats of different body-weights. The rats used in

Table I. *Effects of oestrogenization on body-weight and reproductive organs of adult male rats*

Nos.	No. of rats	Stilboestrol implanted (mg.)	Duration of observation or treatment (days)	Average body-weight		Average weight of	
				At start (g.)	At end (g.)	Testes (g.)	S.v. (g.)
<i>Control groups</i>							
306-310	5	None	13	140	157	1.73	0.135
86-90	5	None	20	116	167	1.900	0.344
26-30	5	None	20	132	184	1.950	0.367
205-209	5	None	20	142	179	2.00	0.378
290-294	5	None	20	150	192	1.81	0.443
56-60	5	None	20	165	203	2.210	0.438
<i>Oestrogenized groups</i>							
177-180	4	30	6	125	131	1.31	0.044
131-135	5	30	5	143	137	1.62	0.054
187-190	4	30	5	—	191	1.73	0.178
121-125	5	30	10	116	106	1.22	0.087
250-254	5	30	10	225	192	1.85	0.173
76-80	5	15	20	108	117	0.944	0.049
126-130	5	30	20	113	120	0.590	0.035
21-25	5	30	20	133	136	0.686	0.045
51-55	5	30	20	138	150	0.495	0.051
200-204	5	30	20	141	135	0.526	0.033
81-85	5	30	20	106	110	0.440	0.038

these experiments showed considerable variation, particularly in the weights of the accessory organs, but the effect of oestrogenization was quite unmistakable. In rats of 100-140 g. body-weight the seminal vesicles, still comparatively small at the beginning of the experiment, were reduced to about 50 mg. after 5 or 6 days' oestrogenization. The testes, as already found [Deanesly, 1939], showed a progressive reduction in size and disorganization and disappearance of the germinal epithelium during the 20-day experiments. It is clear that stilboestrol in tablet form exercises an immediate effect in suppressing the gonadotrophic secretion of the pituitary gland.

Oestrogenization and injection over 10 days

Various gonadotrophic preparations were tested in three types of experiment to determine approximately the dosage which would maintain

Table II. *Oestrogenization and injections over 10 days*

Nos.	No. in group	Extract injected	Daily dose (mg.)	Average body-wt.		Average weight of	
				At start (g.)	At end (g.)	Testes (g.)	S.v. (g.)
96-100	5	AP61B	1	116	121	1.966	0.534
91-95	5	„	2	114	120	2.02	0.567
111-115	5	AP61C	2	132	132	1.127	0.047

the normal size and secretion of the reproductive organs in oestrogenized males. Table II shows apparently that full maintenance can be obtained in a 10-day test by means of horse pituitary extract (AP61B) in a daily dosage of 1 or 2 mg. Sections through the testes of these rats, however, indicated that their fertility had not been maintained; much of the germinal epithelium seemed normal, but the actual spermatozoa had been extensively damaged and it was doubtful if new ones were being produced. It may be noted that if a gonadotrophic substance in a particular dose is adequate to maintain the normal size of the reproductive organs in an experiment of this kind, then twice that dose may not materially increase the size of the testes or seminal vesicles, and in certain cases may actually lead to a decrease, as shown by other experiments (Tables III and V). Table II shows that in this test AP61C, the prolactin fraction of a horse pituitary extract failed to prevent the regression of the testes and seminal vesicles.

The data given in Tables I and II show that so far as the size of the seminal vesicles is concerned, a 10-day period of oestrogenization and injection is adequate to allow a clear-cut result. As regards the size of the testis, however, a longer experimental period seems to be desirable.

Oestrogenization and injection over 20 days

In the next set of experiments (Table III) oestrogenization was prolonged for 20 days. Most of the rats of 135 g. or over showed little gain in weight during the experimental period; the gonadotrophic extracts injected daily did not replace the growth-promoting activity inhibited by the stilboestrol. As regards the reproductive organs, it will be seen that horse pituitary extract AP61B was again partially effective in a daily dose of 0.5 or 1 mg., while doses of 2 mg. and 5 mg. stimulated abnormal growth of the reproductive organs in relation to the body-weight of the rat. Examination of testis sections from the rats receiving horse pituitary indicated considerable variation in the response, corresponding generally to the individual weights of the reproductive organs. Daily dosage of 0.1 mg. and 0.25 mg., though partially preventing testis

shrinkage, proved inadequate for maintenance of the interstitial cells and the tubules were considerably disorganized. The group receiving 0.5 mg. daily showed much variation, but on an average the testes were better

Table III. *Effect of concurrent oestrogenization and injections of gonadotrophins over 20 days*

Nos.	No. in group	Extract injected	Daily dose (mg.)	Average body-wt.		Average weight of	
				At start (g.)	At end (g.)	Testes (g.)	S.v. (g.)
31-35	5		0.1	135	135	0.813	0.053
36-40	5	Horso	0.25	116	144	1.421	0.134
41-45	5	pituitary	0.5	136	143	1.610	0.401
1-5	5	extract	1.0	140	146	1.941	0.720
62-65	4	AP61B	2.0	112	115	2.27	0.923
66-70	5		5.0	118	122	2.36	1.070
71-75	5	Horso pituitary extract AP61C	2.0	115	121	1.61	0.127
6-10	5	Pig pituitary extract AP74D	2.0	121	123	0.622	0.044
11-15	5	Ox pituitary extract AP81B	5.0	150	142	0.800	0.087
302-304	3		0.0025	142	123	0.680	0.061
305-309	5		0.005	140	137	1.99	0.171
320-324	5	Urine of	0.015	138	133	2.150	0.403
325-329	5	pregnancy	0.025	131	128	2.450	0.350
295-299	5	extract	0.025	143	153	1.934	0.669
285-289	5	UP27 ¹	0.050	142	153	2.410	1.106
210-214	5		0.100	138	144	1.820	1.106
215-219	5		0.250	137	156	2.260	1.140
16-20	5		0.500	135	145	1.540	0.816

maintained. With dosages of 1-5 mg. daily the stimulation of the interstitial cells tended to be excessive as shown by the size of the accessory glands. In section the cells were more prominent than in the normal testis and showed much cytoplasm. Even in the last two groups, however, in which the testes were regaining their normal size, the germinal epithelium showed some signs of damage. The rats receiving 2 mg. daily were probably still fertile but 5 mg. daily in some cases caused abnormal oedema of the testes. AP61C, the prolactin fraction, though ineffective in the 10-day test, here prevented the full regression of the testes and reproductive organs, owing presumably to the traces of gonadotrophic activity present. AP74D, a gonadotrophic extract of pig pituitary gland,

¹ 1 mg. UP27 contained 200 international units of chorionic gonadotrophin.

however, had no distinguishable effect in a 2-mg. daily dosage. Ox pituitary gland extract AP81B apparently partially maintained testis size, but did not prevent the regression of the seminal vesicles. These last results were disappointing, since both the last two extracts are predomi-

Table IV. *Effect of concurrent oestrogenization and injections of pregnant mare serum (PMS26) over 20 days*

Nos.	No. in group	Daily dose (I.U.)	Average body-weight		Average weight of	
			At start	At end	Testes	S.v.
			(g.)	(g.)	(g.)	(g.)
310-314	5	0.5	143	116	1.08	0.055
280-284	5	1	142	145	2.15	0.398
275-279	5	2	142	149	1.90	0.574
235-239	5	4	136	149	2.35	0.952
245-249	5	8	136	147	2.24	1.230
240-243	4	26	136	148	2.47	1.160

nantly 'luteinizing' and might have been expected to maintain the secretion of the interstitial cells of the testis. The urine of pregnancy extract UP27 in very small doses proved adequate for maintenance of the size and secretory function of the testes—in fact a daily dose as low as one-fortieth of a milligram stimulated the interstitial cells to hypernormal activity judging by the size of the seminal vesicles. It will be noted that with 20 times this dose neither the testes nor the seminal vesicles were abnormally large at the end of the experiment. In the group receiving 0.05 mg. daily the germinal epithelium, with the exception of some of the spermatozoa, seemed to be well maintained. It could not be stated with certainty, however, that the rats would have been fertile.

Pregnant mare serum proved to be highly effective, one or two international units daily being adequate for gross maintenance of the reproductive organs. Larger amounts caused further increase in the testes and seminal vesicles, but allowing for normal variation within the groups 8 I.U. daily had not much more effect than 4 I.U. With both these doses the reproductive organs were larger than in the corresponding normal control group and above normal size in relation to the body-weights of the animals. With the three highest doses of pregnant mare serum the average body-weight increase in 20 days was 12 g., indicating that slight growth was still taking place. Sections through the testes of the group receiving 1 I.U. daily indicated that the germinal epithelium, with the exception of some of the spermatozoa, had been satisfactorily maintained.

Oestrogenization over 20 days with injection over the last 10

Some gonadotrophic preparations evoke immunity to their action very rapidly (see Hamburger [1938] in case of mare serum extracts) and it

was thought that the results of the 20-day injections described above might have been interfered with by the animals becoming resistant during the latter part of the experimental period.

As a shorter period of oestrogenization was not thought to be sufficient, it became necessary, in order to shorten the period of injection, to introduce the principle of restoration as well as maintenance of the reproductive organs.

A third set of experiments (Table V) was therefore completed in which stilboestrol tablets were implanted for 20 days but gonadotrophic injections were given only during the last 10 days. The results, taken with

Table V. *Effect of oestrogenization for 20 days and injection of gonadotrophins over the last 10 days*

Nos.	No. in group	Extract injected	Daily dose (mg.)	Average body-wt.		Average weight of	
				At start (g.)	At end (g.)	Testes (g.)	S.v. (g.)
107-110	4	Horse	1.0	106	109	1.413	0.473
101-105	5	pituitary	2.0	111	128	1.920	0.682
225-229	5	extract	2.0	138	141	1.660	0.407
230-234	5	AP61B	4.0	137	141	1.414	0.364
156-160	5	Pig pituit.	2.0	181	166	0.978	0.106
161-165	5	ary extract	5.0	214	181	1.117	0.090
166-170	5	AP74D	10.0	206	177	1.055	0.067
220-224	5	Urino of	0.1	140	143	1.190	0.221
141-145	5	pregnancy	0.5	208	184	0.981	0.130
136-140	5	extract	1.0	214	192	1.102	0.206
146-150	5	UP27	2.0	197	170	0.811	0.142

the data in Table I, show that when the reproductive organs have been regressing for 10 days, they can be restored to normal size by 10 daily injections of horse pituitary extract in spite of continued oestrogenization. Given in this way, however, doses of 40 mg. and 20 mg. respectively of AP61B produce smaller testes and seminal vesicles than the same total doses spread over the whole 20 days of the experiment. The pregnancy urine extract also was not as effective in restoring testicular activity as in maintaining it (Tables III and V). The pig anterior pituitary extract AP74D failed to restore, and probably even to maintain, the size of the seminal vesicles and testes (Table V).

In general these results show that restoration over a period of 10 days is more difficult than maintenance over 20 days, and they do not lend any support to the view that the effectiveness of 20 daily injections is compromised by antihormone formation.

DISCUSSION

The results indicate that gonadotrophic extracts will maintain the size of the male reproductive organs in rats in which the activity of the pituitary gland is inhibited by implanted oestrogen. It is possible, moreover, to obtain a graded response by the use of different doses of gonadotrophic extract on rats of similar body-weight at the time of oestrogen implantation (Tables III and IV), i.e. there is a relation between dose and response. The results do not, however, suggest that such oestrogenized male rats would be suitable for the assay of gonadotrophic preparations of unknown strength. There is always considerable individual variation in the size of the accessory organs and though there is no reason to suppose that this is greater than in the case of other test-objects, the fact that their response to injection in the oestrogenized animal is an indirect one, increases the variables inherent in the test. Moreover, while size changes in the accessory glands may reasonably be considered as an uncomplicated indication of interstitial cell activity, size changes in the testes are obviously dependent on a variety of factors. It is known that in hypophysectomized animals spermatogenesis may be maintained or even restored by androgens, and it may be assumed that in these experimental rats functional spermatogenesis is affected indirectly by the condition of the interstitial tissue as well as directly by the gonadotrophic injections. As regards intertubular tissue, changes in the interstitial cells cannot be supposed to affect testis-weight *per se*, but intertubular fluid accumulation seen in some of the injected animals must materially do so.

General histological study of the experimental material indicates that the maintenance of the germinal epithelium by means of gonadotrophic extracts is much less simple than maintenance of the interstitial cells. Even where the tubules look superficially normal, the spermatozoa are often observed to be disintegrating and the later stages of spermatogenesis are not proceeding normally. Large testes, associated with large accessory glands, might be found completely sterile on histological examination, having perhaps been over-stimulated. On the whole the testes from experimental rats appearing most normal were those injected from the beginning of the 20-day period with horse pituitary extract, pregnant mare serum or urine of pregnancy extract. Pituitary gland extracts of a predominantly 'luteinizing' character proved almost useless to maintain the testes.

SUMMARY

The subcutaneous implantation of two 15 mg. tablets of diethylstilboestrol into male rats of body-weight 100-140 g. causes rapid regression of the testes and accessory glands.

Rats thus treated respond readily to gonadotrophie extracts of horse pituitary gland, urine of pregnant women and serum of pregnant mares, but not to those of pig and ox pituitary gland.

Some of the variables pertaining to the test are examined and diseussed.

Our best thanks are due to Dr. F. G. Young who prepared the pituitary extracts.

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QUANTITATIVE STUDY OF THE EFFECTS OF IMPLANTING TABLETS OF OESTROGENS AND ANDROGENS IN RATS

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THE prolonged administration of oestrogenic substances to male animals produces very marked effects. The more interesting of these effects, notably those on growth and on the gonads, are apparently exerted indirectly by depression of hypophyseal activity. Much of the work has been carried out on the male rat, in which stunting of the body and atrophy of the testes can readily be obtained by prolonged administration of oestrogens. This capacity to depress pituitary activity is seen both in the naturally-occurring oestrogens and in the synthetic oestrogens such as diethylstilboestrol [Noble, 1939].

It is already known that substances which have multiple properties in common may not show them equally intensely. As an extreme example it may be recalled that methyl testosterone is highly androgenic and slightly progestational, while progesterone is highly progestational and faintly androgenic. Also, evidence is already appearing that the various multiple activities of the oestrogens are not equally intense in all oestrogenic substances. Thus, the theoretical possibility exists that further work will result in the preparation of substances which, for instance, depress pituitary activity but do not cause the characteristic changes in the reproductive tract of the female, or again, of substances which depress only one pituitary function. Such substances might be of great practical as well as theoretical interest, since the use of existing oestrogens to correct an overactivity of the pituitary gland (overgrowth, for instance) would lead to unwanted hyperplasia in the reproductive tract.

Detailed comparison of different substances can only be made by accurately assaying all their known activities, and it is important that a full range of appropriate methods should be available. Accurate methods have already been worked out for many of the activities, but quantitative study of the antigrowth and antigonad effect has not yet progressed far. Both of these are essentially long-term effects and it has already been shown that administration of the substances by the tablet implantation technique produces the result effectively and with the minimum of labour [Deanesly, 1939]. The present paper provides a quantitative basis for the simple comparison of the antipituitary activity of oestrogenic substances.

EXPERIMENTAL

*Implantation of oestrogens in young male rats**For long periods*

If young male rats are hypophysectomized at 40–50 g. body-weight, growth proceeds fairly normally to a weight of 70–80 g. and thereafter ceases abruptly. The effect of early oestrogenization is rather different, as pointed out by Noble [1939]. Fig. 1 shows that although the growth rate of immature rats is immediately depressed by oestrogenization, growth does not cease until the rats are over 120 g. With tablets of about 15 mg., oestradiol has a rather more marked effect than oestrone; this result might be expected since oestradiol has greater biological activity, and it has a greater rate of subcutaneous absorption [Deanesly, 1939].

Table I. *Depression of growth rate in male rats by oestrogens*

No. of rats	Average body-wt. at implantation (g.)	Substance implanted	Average amount implanted per rat (mg.)	Average body-wt. three months later (g.)
20	40	—	—	186
5	44	Oestrone	14.7	152
5	48	Oestrone	50.9	124
20	46	Oestradiol	14.0	118
4	77	Stilboestrol	15.2	116

With much larger tablets of oestrone (average 51 mg.), the growth inhibition is nearly as great as with the smaller tablets of oestradiol. The anti-growth effect of the implantation of stilboestrol tablets is substantially similar, as found by Noble [1939] who tested the effect of various synthetic oestrogens and carcinogens on the growth rate.

Four rats averaging 77 g. received 15 mg. tablets of stilboestrol; Fig. 1 shows the immediate depression of the growth rate. These rats were killed after 96 days as the tablets were tending to disappear, since the solubility of stilboestrol is about ten times that of oestrone. The depression of the growth rate is about the same as that produced by oestradiol and takes effect in comparatively young rats. It appears likely that the anti-growth effect of the oestrogens is to some extent exerted directly, since it is shown at a time when the pituitary gland is unnecessary for growth. Whether this direct effect is due to influence on the tissues themselves or to decreasing the food intake of the animal is not clear.

The general effects of oestrogen administration in tablet form to 40 immature male rats correspond closely to those already described for adult rats [Deanesly, 1939]. Growth of the gonads and accessory organs was inhibited and the testes and prostate remained very small (about 70–200 mg.); the seminal vesicles which respond to oestrogen administration by fibrosis enlarged to a limited extent (88–160 mg.) but showed no

secretory activity. The adrenals were variable but on the whole larger than in the controls. The rats showed less tolerance for the oestradiol than for the oestrone; pituitary gland tumours developed in five rats after about 9 months implantation. Only two out of twenty-four rats survived for a year.

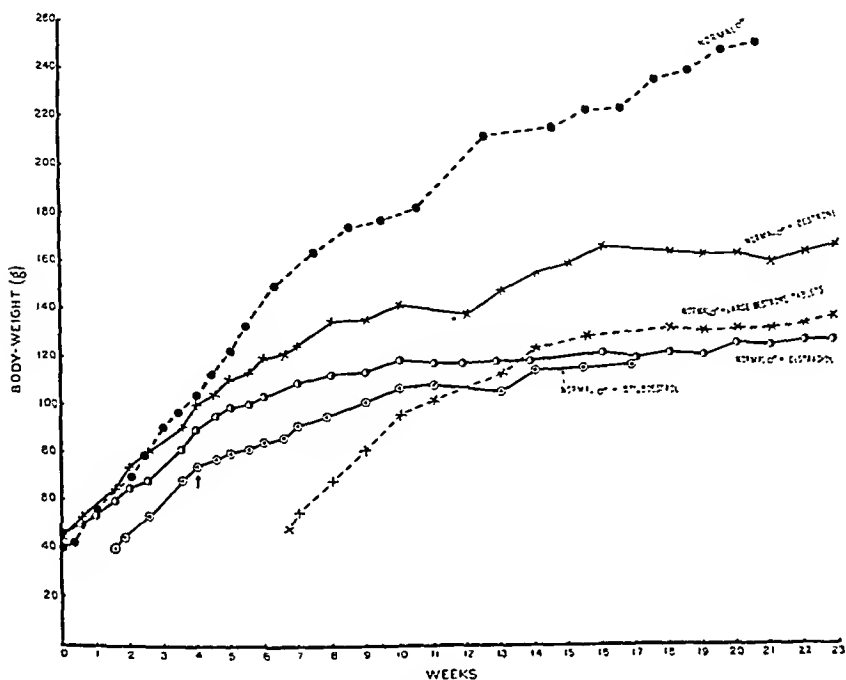


FIG. 1

FIG. 1. Depression of the growth rate of immature male rats by the administration of oestrogens. Except for the rats receiving stilboestrol, in which the time of implantation is marked with an arrow, tablets were implanted at the beginning of the period of observation. Other details in text.

Implantation for short periods

A series of experiments was carried out to see if a simple quantitative 15- or 30-day test could be devised for studying pituitary gland inhibition. The results showed that marked changes could be produced during these periods in rats of a suitable size, and a quantitative comparison could therefore be made between an unknown substance and a known oestrogen.

Four groups of five rats averaging 48–49 g. body-weight were selected and oestrone tablets of about 16 mg. were implanted subcutaneously in half of the animals, the others remaining as controls. One normal and one control group were killed after 15 or 30 days, and Table II gives the body-weights and weights of the reproductive and endocrine organs at autopsy. It is clear from Fig. 1 that in short tests of this kind on immature

rats the body-weight could not be used as an index of pituitary gland inhibition, and the extent of the difference between the control and the 15-day experimental group is probably fortuitous. The reproductive organs and the pituitary glands in the oestrone groups, however, show well-marked differences in weight from those in the normal groups.

Table II. *Effects of 15 and 30 days' pituitary gland depression by oestrone in rats: comparison of the average weights of reproductive and endocrine organs in normal and treated males*

Group	1st wt. (g.)	Last wt. (g.)	Days	Testes (g.)	Prostate (mg.)	S.v. (mg.)	Pituitary (mg.)	Adrenals (mg.)
I Normal	48	107	15	1.05	73	26	3.6	20
Ia Oestrone	49	70	15	0.22	37	28	4.6	22
II Normal	49	122	30	1.56	172	110	5.0	25
IIa Oestrone	49	125	30	0.76	60	51	10.0	28
III Normal	111	—	—	1.50	158	109	4.0	21
IIIa Oestrone	110	121	15	1.36	115	42	8.5	36
IV Normal	114	181	30	2.41	509	488	5.0	27
IVa Oestrone	118	143	30	0.79	113	48	13.0	40

Table II also records similar tests on groups of young adult rats in which implantation of 16 mg. oestrone tablets for 15 or 30 days caused shrinkage of the testes and accessory organs and enlargement of the adrenals. Group III shows the approximate sizes of the latter at the beginning of the experiment, and Group IV the weights in similar rats 30 days later. Comparing these with Groups IIIa and IVa receiving oestrone, it is clear that significant differences in the size of the organs have been produced.

Another 30-day test on small groups of young rats showed that with oestrone tablets averaging respectively for each group 12.4, 9.4 and 5.9 mg., the extent of the pituitary gland inhibition was the same throughout. In earlier experiments with larger rats [Deanesly, 1939] it was found that very small amounts of oestrogens were adequate to produce almost the maximum inhibition for that substance.

Implantation of oestrogens in castrated rats

Ten rats were castrated at about 40 g. and five of them were given 15 mg. tablets of oestrone subcutaneously. The surviving three rats in each group were killed one year later. Fig. 2 shows that the depressing effect of oestrogens on growth is apparent in castrate as in normal male rats, although the growth of the former is already depressed by comparison with that of the normal animal [cf. Rubinstein, Kurland & Goodwin, 1939]. The growth of castrated rats with oestrone tablets is rather more depressed than that of normal rats with (15 mg.) oestrone tablets of similar size.

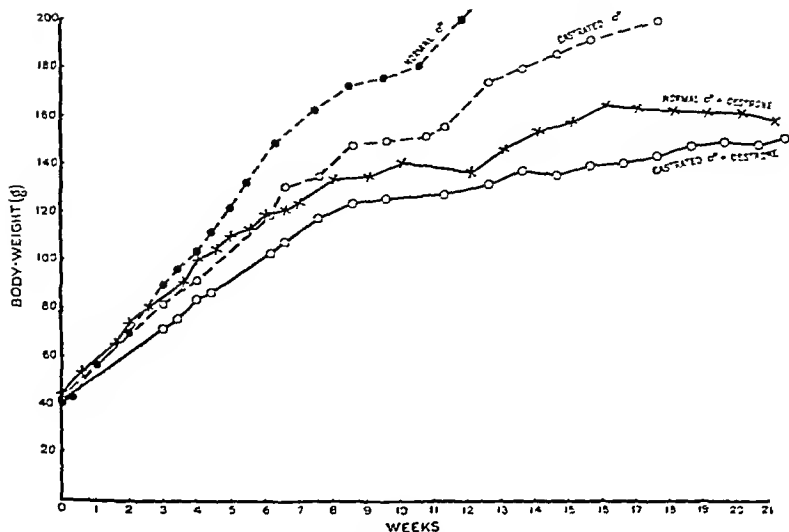


FIG. 2

FIG. 2. Effect of castration on the growth rate of normal and oestrogenized immature male rats. Castration and tablet implantation were carried out at the beginning of the period of observation.

Implantation of oestrogens or androgens in young female rats

Noble [1939] has shown that oestrogens in tablet form will inhibit growth in female as well as in male rats; the adrenals and uterus enlarge but the ovaries become atrophic in such animals. Similar results were obtained in the present experiments, begun in January 1939; twenty rats received oestrogen implantations but a number of them died less than a year later. Those surviving more than 9 months showed pituitary and mammary gland tumours. Fig. 3 shows that growth was depressed by oestrogens to about the same extent in females as in males. In an earlier experiment the subcutaneous implantation of tablets of testosterone propionate inhibited the growth of normal female rats to about the same extent as does oestrone (Fig. 3). After 81 days implantation and absorption of about 15 mg., this group of rats had atrophied ovaries but well-developed uteri. The pituitary glands were not enlarged. Similar results were obtained by Rubinstein *et al.* [1939] and Kochakian [1940]; the latter makes no reference to the effects of continued sex hormone injection on the pituitary gland.

Simultaneous implantation of oestrogen and androgen in young male rats

Both in birds and in mammals there is mutual inhibition between androgens and oestrogens. Thus the comb growth promoting effect of

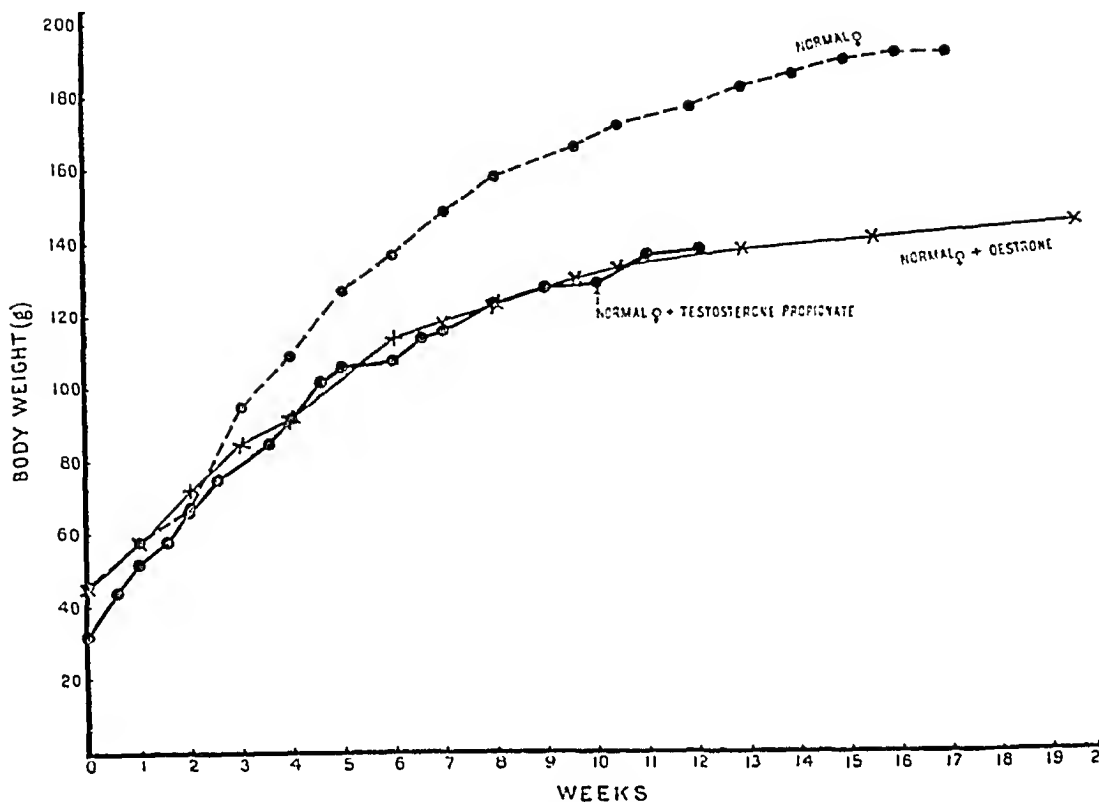


FIG. 3

Fig. 3. Depression of the growth rate of immaturo female rats by the administration of testosterone propionate or oestrone. Tablets were implanted at the beginning of the period of observation.

androgens in capons can be suppressed by the simultaneous administration of oestrogens [Emmens & Bradshaw, 1939]. In rats and mice the cornification of the vagina in response to injection of oestrogen can be prevented by the simultaneous injection of androgen. In male mammals protection from the effects of oestrogens can be obtained if androgens are given simultaneously. The literature on this last subject is extensive as regards the testis and accessory organs and need not be reviewed here. The protection of the male from the pituitary-depressing action of oestrogens by the injection of androgens seems to have been little investigated. Such a study is inherently complicated, since androgens themselves may cause depression of pituitary activity in the male [Rubinstein *et al.*, 1939]. Wolfe & Hamilton [1937] made a histological study of the pituitary glands of male rats injected simultaneously with oestrone and testosterone propionate. They found that the percentage of chromophobes did not increase, and weight and histological changes were not as marked as in rats receiving the same dose of oestrone only. They concluded that the androgen had partly inhibited the effect of the oestrogen.

The following experiment was carried out to extend this kind of investigation to a study of the functional activity, especially the somatotrophic activity, of the pituitary gland.

Seven groups of five rats each were used, the body-weight when observations started averaging between 50 and 60 g. Two groups were kept as controls, one group was given diethylstilboestrol only, and one testosterone propionate only, while the remaining three groups received tablets of both substances. Stilboestrol was administered to each rat receiving it by the subcutaneous implantation of one 15 mg. tablet. The tablets of testosterone propionate varied somewhat in weight, but they averaged rather over 20 mg. From what is known of the absorption rates of these two substances administered in tablet form [Deanesly & Parkes, 1938] it was anticipated that the stilboestrol tablets would be absorbed in the course of about three months, whereas the testosterone propionate tablets would take considerably longer. This expectation was realized, since when the rats were killed 85 days after the implantations were made the tablets of stilboestrol had disappeared while those of the androgen were still present, but reduced to about half their previous size.

The growth curves for the four categories of rats are shown in Fig. 4. The curves for the control and stilboestrolized rats are similar to those given earlier in this paper. Moreover, the difference in the growth rates of the control and androgenized rats are similar to those observed by Rubinstein *et al.* [1939], and indicate slight growth inhibition. The growth curve for the fifteen rats receiving both oestrogen and androgen is most instructive and shows clearly that the depression of the somatotrophic activity of the pituitary gland produced by stilboestrol can be at least partially prevented by the simultaneous administration of androgen. During the first three weeks after implantation the rats receiving testosterone and stilboestrol increased in weight twice as rapidly as those receiving stilboestrol only. The latter stabilized at a weight of about 110 g., a result in keeping with those recorded earlier in this paper. The rats receiving the double implantation stabilized at about 140 g., a weight which is not reached by heavily stilboestrolized rats. Nevertheless, the inhibition of the antigrowth action of oestrogens was only partial, since the body growth of rats receiving both substances fell far behind that of the controls and androgen-treated animals.

The condition of the testes of the rats in the various groups indicated that the depression of gonadotrophic activity had been similar to that of somatotrophic activity. The untreated animals all had large testes in full spermatogenic activity; those receiving stilboestrol only had very small testes, in which both the tubules and the interstitial cells showed the full atrophy previously described [Deanesly, 1939] in oestrogenized animals.

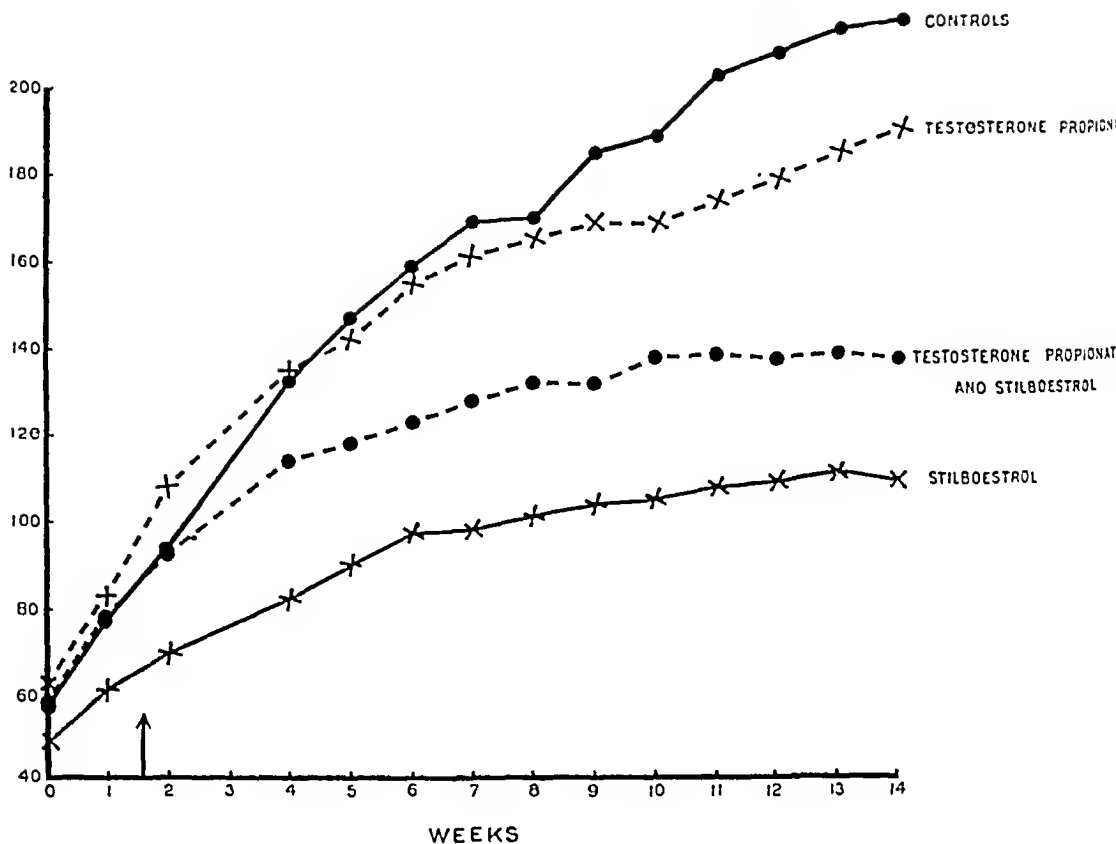


FIG. 4

FIG. 4. Effect of the simultaneous administration of androgen on the anti-growth action of oestrogen. The time of implantation of tablets of testosterone propionate and stilboestrol is shown by the arrow.

The typical intra-tubular oedema was also present. The testes of the rats receiving the double implantation were somewhat variable, but the most atrophic were considerably less so than those of the rats receiving oestrogen only. The less atrophic were moderately large, and fully formed, and contained spermatozoa. Histologically, therefore, the testes had been substantially protected from the antipituitary effects of oestrogen by the simultaneous administration of testosterone propionate. Whether this effect is due wholly to protection of the pituitary gland from the effects of the oestrogen is not clear, since androgens have a considerable power of maintaining spermatogenesis at least for a time, when the pituitary gland has been surgically removed [Walsh, Cuyler & McCullagh, 1934]. Another feature revealed by the histological examination of the testes was the comparative smallness and quiescence of the testes of the rats receiving testosterone propionate only. Spermatozoa were present in all, but the testes as a whole, and the individual tubules, were only of

moderate size, and spermatogenesis was not very active. This observation recalls the result of Bottomley & Folley [1938] who found that administration of testosterone caused testicular atrophy in young guinea-pigs, and emphasizes the interest of the fact that the strongly inhibitory effect of oestrone and the mildly inhibitory effect of testosterone on the testes are antagonistic, not additive, when both are administered together.

SUMMARY

Administration of oestrogens to male rats of 40–50 g. body-weight causes immediate retardation of the growth rate, but growth does not cease till a weight of 120–130 g. is reached.

Changes in the testes and accessory organs following oestrogenization are sufficiently advanced at 15 days to make possible an estimate of the antipituitary activity of an unknown substance under test.

The growth rate of castrated rats is lower than that of normal male rats, and is further depressed by oestrogenization.

The growth rate of female rats is depressed by the administration of either oestrone or testosterone.

The antigrowth and antigonadotrophic effect of stilboestrol in male rats can be inhibited partially by the simultaneous administration of testosterone.

We are much indebted to Messrs. Boots Pure Drug Company for the supply of tablets of diethylstilboestrol, to Messrs. Ciba for the testosterone propionate and to Organon Laboratories for the oestrone and oestradiol tablets.

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abundant, but a number of the dividing cells were pushed into the lumen and were undergoing degeneration.

Discussion

The hypophyseal control of the gonad in birds has been described by Hill & Parkes [1934*b*]. They found that in Brown Leghorns regression of the comb follows immediately after removal of the hypophysis, and the rate of regression is much the same as that in the castrated adult cock. This indicates that testicular secretion ceases abruptly after the extirpation of the hypophysis. The lack of secondary sexual characters in the pigeon makes it difficult to ascertain the immediate effect of hypophysectomy on the sex glands. However, basing conclusions on the weight and histological changes of the gonad, it is likely that the testis begins to regress immediately after removal of the pituitary.

Pigeon 0-529 offers the best example of the abrupt cessation of ovarian activity following hypophysectomy in the female. In this bird eggs were seen in the oviduct at the time of operation. The bird died 12 days later and the eggs, being in a state of liquefaction, were recovered from the oviduct *post mortem*. This suggests that the failure to lay the ovulated eggs was caused by the withdrawal of ovarian secretions on which the normal functioning of the oviduct depends.

It has been established that both the gonad and the thyroid gland are dependent upon the anterior pituitary for their normal functioning. But the sensitivity of these two organs to removal of hypophyseal control is different. We have found in hypophysectomized pigeons that the decrease in the weight of the thyroid is not as rapid as in that of the testis, and the hypothyroid type of plumage can be obtained only in the long-surviving birds. This suggests that the thyroid atrophies rather gradually. Some evidence from two hypophysectomized Brown Leghorn cockerels supports this view. In the male Brown Leghorn the regression of the head furnishings and the colour changes in the breast feathers are two characteristic manifestations of hypopituitarism which indicate hypofunction of the gonad and thyroid respectively.

It was recorded by Hill & Parkes, and has been noted in the writer's experience, that the comb atrophy begins before the plumage change after total hypophysectomy of a Brown Leghorn cock, or may occur in its absence when hypophysectomy is incomplete.

The gonads of hypophysectomized pigeons can be maintained or restored by the administration of either horse or sheep pituitary extract. Hill & Parkes [1935], on the other hand, reported that neither ox nor horse pituitary extract could prevent gonad atrophy in hypophysectomized Brown Leghorn cockerels. Species differences in gonad reaction to various

pituitary preparations are well known to occur but, as mentioned in the introduction, it is not clear to what extent these differences are due to the qualitative properties of the extracts.

The most informative part of this study is perhaps that relating to the effect of male hormone on the testes of the normal and hypophysectomized pigeons. It has been repeatedly shown by previous workers that male hormone suppresses hypophyseal activity in mammals and indirectly causes regression of the gonad. The same effect has now been shown to occur in pigeons.

As regards the gonadergic effect of androgens, it has been demonstrated in the present study that testosterone maintains spermatogenesis in newly hypophysectomized pigeons and may also reinitiate testicular growth when administration is delayed. This affords good evidence that the effect is exerted directly on the seminiferous tubules and not indirectly by the maintenance of scrotal function. Attention may here be called to the extraordinary paradox that androgens are capable, at least for a time, of preventing the atrophy of the tubules caused by removal of the pituitary, but not the atrophy which follows the injection of androgens to the normal male and which is caused, apparently, by the depression of pituitary activity.

The effect of male hormone on ovarian activity of the female needs to be further emphasized. In the present experiments the ovarian function in hypophysectomized female pigeons has been considerably and consistently stimulated by testosterone. This agrees with the findings of Starkey & Leathem [1938] and Salmon [1938] on immature mice. In the opinion of the latter author the effect is due to stimulation of the pituitary gland of the female, but it seems that the gonadergic activity of the androgen itself may provide an adequate explanation.

SUMMARY

(1) Hypophysectomy in the pigeon caused rapid regression of the gonads and accessory sexual organs. Histologically, the testes of hypophysectomized birds showed reduction in tubule size and interruption of spermatogenesis. The intertubular tissue was relatively increased, but the hormone-producing cells no longer functioned, as shown by the extreme atrophy of the vasa deferentia. In the ovaries of hypophysectomized females there were no healthy-looking follicles of more than 1 mm. in diameter. The oviduct was atrophic and lacked glandular structure.

(2) Replacement therapy with pituitary extracts in hypophysectomized birds proved effective. Pituitary preparations with high follicle-stimulating activity, such as those from horse and sheep pituitary glands, were more effective in the restoration of gonadal activity than ox pituitary

extract containing mainly luteinizing activity. Administration of horse pituitary extract either immediately after hypophysectomy or later on proved to be equally effective.

(3) Injection of testosterone to normal sexually mature pigeons caused damage to the testes. Administration of the same hormone to hypophysectomized birds resulted in the maintenance of gonadal function. The deleterious effect of male hormone on the testes of normal birds is probably exerted indirectly through the pituitary, while its stimulating effect in hypophysectomized birds is almost certainly direct.

It is a great pleasure to acknowledge my indebtedness to Sir Henry Dale and the Medical Research Council for the hospitality of the National Institute for Medical Research Farm Laboratories, to Mr. R. E. Glover, Superintendent of the laboratories, for his helpful assistance, to Dr. A. S. Parkes for his continued interest and advice, and to Dr. Zuckerman for statistical assistance.

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MORPHOLOGICAL RELATIONSHIP BETWEEN TESTICULAR NERVES AND LEYDIG CELLS IN MAN

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It is well known that the connective tissue of the testicular intertubular spaces contains a variable number of the so-called Leydig cells, epitheloid elements of which the structure has been minutely described but whose function is still unknown.

Since Berthold's [1849] testis transplantations in hens (an experiment which had already been carried out by John Hunter in 1792) an endocrine function has been rightly ascribed to the testis; this conception being extended by the experiments of Brown-Séquard [1889] and numerous subsequent workers.

Biologists have disagreed as to whether the spermatogenic epithelium with the Sertoli cells, or the Leydig cells of the intertubular tissue, were responsible for the endocrine function. Supporters of the latter theory were Bouin & Ancel [1903], Steinaeh [1910], Sand [1933], Lipschütz [1924] and others. In fact most physiologists and morphologists now adhere to the theory that the Leydig cells are responsible for the hormone production of the testis. A detailed discussion of this subject is to be found in a monograph by Sand [1933] and reference may be made to other papers by the same author [1919, 1921, 1923].

Study of the Leydig cells therefore occupies a very important position in endocrine research on the testis. Our knowledge of the histology and physiology of these cells is, however, very incomplete, as is also that of the microscopic anatomy of the testicular blood-vessels and nerves. The reason for this ignorance is presumably that histologists have for generations been fascinated by the tremendous problems of spermatogenesis. Observation of the structures comprising the interstitial framework of the gland was chiefly confined to the Leydig cells; then to the blood-vessels and lymphatics; and finally to the nerves, which are mentioned only in passing even in the larger monographs on the testis. The current description of these nerves can be summed up in three sentences. Only a few nerves enter the testis. Some are vasomotor and others are sensory. Their peripheral ramifications are unknown.

We have carried out, during the past 10 years, a comprehensive histo-

logical and physiological examination of the testicular material provided by sexually abnormal men castrated by authority of the Danish Sterilization Acts, 1929 and 1935.

Obviously, the study of the Leydig cells, as a possible factor in bringing about the psycho-sexual disorders, was emphasized in our histological work [Sand & Okkels, 1936*a*, *b*, *c*, 1938]. A review of this work is outside the scope of the present paper. It may suffice to mention that the frequent observation of regressive alterations of the tubules—both in normal and in sexually abnormal cases—and the occurrence of hyperplastic changes of the Leydig cells, led us to examine with special care the interstitial stroma of the testis. Working systematically with serial sections, we were struck by the fact that the testicular nerves are so much larger and more numerous than one would expect as judged by the scanty literature on the subject. And when we found evidence of a morphological relationship between the nerves and the Leydig cells, we naturally paid special attention to the interesting problems involved. Preliminary reports of these findings have been given on two occasions [Okkels & Sand, 1938, 1939*a*]. Since then our observations have been extended to such a degree, and the results have proved so consistent, that we now propose to give a more comprehensive statement of our work.¹

The fact that we have observed a close morphological relationship between the Leydig cells and the testicular nerves seems to be of significance. These observations may help to explain several phenomena which are linked to the sexual abnormalities, and they may also be important in the histology and physiology of the normal testis.

Material and methods

Our material of about 400 testes can be grouped in equal halves. The first group comprises the testes from about one hundred sexually abnormal individuals, these testes being secured by castration. Immediately after the operation the glands were placed in 10% aqueous formol. After the preliminary fixation the organs were sliced transversely and some of the pieces were transferred to Zenker-formol and potassium bichromate; others were impregnated with osmic acid, while others were kept in neutralized formol for subsequent silver impregnation. The latter material was principally utilized for the present study. From the Zenker-fixed material were cut sections to be stained with iron haematoxylin and Masson

¹ During the preparation of this report the paper of de Amaral [1938] came to our attention; de Amaral has investigated similar phenomena in the ovary. Mention of his paper is inserted in the last part of the present paper. Just before going to print we received the publications of Berger [1922, 1932], whose work we had unfortunately overlooked, showing that he has made observations in the testis similar to those which we have reached independently, and which we now extend.

trichrome stain. The second group of testes from about 100 autopsies were secured a few hours post-mortem and fixed as stated above. This group was regarded as a control group, the subjects being all medically healthy persons killed by traffic accidents or suicide. As the relationship between the nerves and the Leydig cells has been demonstrated regularly in preparations from both groups we shall not make any distinction between the two categories of our material in the present paper.

Paraffin sections were cut in complete series and the following staining techniques were employed:

Silver impregnation. Sections 5-7 μ . The paraffin was removed and the sections were placed in a 2% solution (freshly prepared each time) of 'Protargol' (Merck) for 3 days in an incubator at 35°, rinsed in distilled water and reduced (15 minutes) in a solution of hydroquinone 2 g., formol 5 ml., distilled water 100 ml. (this solution must also be prepared freshly each time). After washing in distilled water the sections were passed into 1% gold chloride for 30-45 minutes. The sections were again washed in distilled water before passing into a solution of acetic acid 100 ml. and glycerine 10 ml. for 1 hour. The sections were then rinsed in distilled water and embedded in paraffin.

Iron haematoxylin. In order to check up on the results of the silver impregnation, alternate sections of the complete series were picked out, mordanted in 3% potassium bichromate solution and stained with Heidenhain's iron haematoxylin. As a rule section numbers 1-2, 4-5, 7-8 and so forth are stained by this method, while numbers 3, 6, 9, &c., were saved for silver impregnation. By comparing silver impregnated sections with the neighbouring preparations stained with iron haematoxylin we were safeguarded against confusing nerves with connective tissue. Details of how to discriminate between the different types of argyrophile fibrils have been published elsewhere [Okkels & Sand, 1939b].

Diagrams of the types of connexion between nerves and Leydig cells are given in the text. Microphotographs of sections prepared by the above methods are shown in Plates I-III.

The testicular nerves and their relationship with the Leydig cells

The nerves reach the organ near the mediastinum testis together with the arteries. The blood-supply of the gland is derived from two sets of arteries. The smaller pursue direct courses through the corpus Highmori and enter the connective tissue which separates the tubuli recti and the summits of the glandular lobules. The larger arteries deviate before entering the mediastinum testis and merge with the dense collagenous strata of the albuginea. Inside this fibrous tunic the arteries run in the deep layer. From time to time branches of very considerable size are

given off into the septula between adjacent bases of the glandular lobuli. From such regions interlobular arteries pursue a straight radial course until they break up in the branches which supply the intertubular capillary bed.

The nerves divide in a similar manner. Only a few slender nerves pass directly through the mediastinum testis. The larger and more numerous nerves follow the arteries running in the deep layer of the fibrous tunic. Like the arteries they twine round the organ and, similarly, branch off into the septula testis. These interlobular nerves are to be found with the greatest regularity in the regions situated radially to the corpus Highmori (Plate I, Fig. 1). It is in these regions that the most conspicuous relations between Leydig cells and nerve tissue are observed.

As stated above, we have been able to demonstrate some of the peculiarities of the testicular nerves by the systematic study of serial sections. Much depends on the proper orientation of the plane in which the sections are cut. It often happens that entire series must be discarded before a sequence of really instructive preparations from a given block is obtained. If, however, one tries hard enough it is always possible, in testes from puberty onwards, to demonstrate the phenomenon of contact between nerves and Leydig cells. As our observations grew more numerous certain features were noted again and again. It became possible to reconstruct a whole range of structural relationships from which emerged a few distinct types of contact between the nerves and the Leydig cells. In short, we were able to condense the observations from thousands of sections into a few general facts. We feel that the description of our results will gain in clarity if we forthwith state our findings in the terms of such generalization.

Immediately after the larger nerves pass into the deep layer of the fibrous tunic they may in places touch elongated groups of Leydig cells. This contact tends to become more pronounced and a certain intermingling occurs until the peripheral regions of the interlobular septula are reached. Inside these partitions the most intense blending is encountered. As the connexion is established between nerves and Leydig cells three stages can be distinguished; three types of contact may therefore be described which differ topographically as well as structurally. The first type (Fig. 2a) is to be found in the region of the hilum or along the nerves immediately after they have entered the fibrous tunic. In this deep vascular layer of the albuginea elongated groups of Leydig cells are regularly present. They may actually touch the nerves and in that case they are often found inside the perineural lymphatic endothelium. It is essentially a type of superficial contact; as a rule, no blending occurs near the corpus Highmori. The second type (Fig. 2b) is characteristically a blend-

ing. Small groups, each consisting of very few Leydig cells, are found embedded in the larger nerves; cross-sections of such nerves show the Leydig cells scattered like raisins in a bun. Topographically this type is most frequently observed near the base of glandular lobuli where the nerves are about to bend into the larger septula. Finally, a third type of connexion between nerves and Leydig cells (Fig. 2c) is met with inside the connective tissue of the interlobular partitions (see also Plate I,

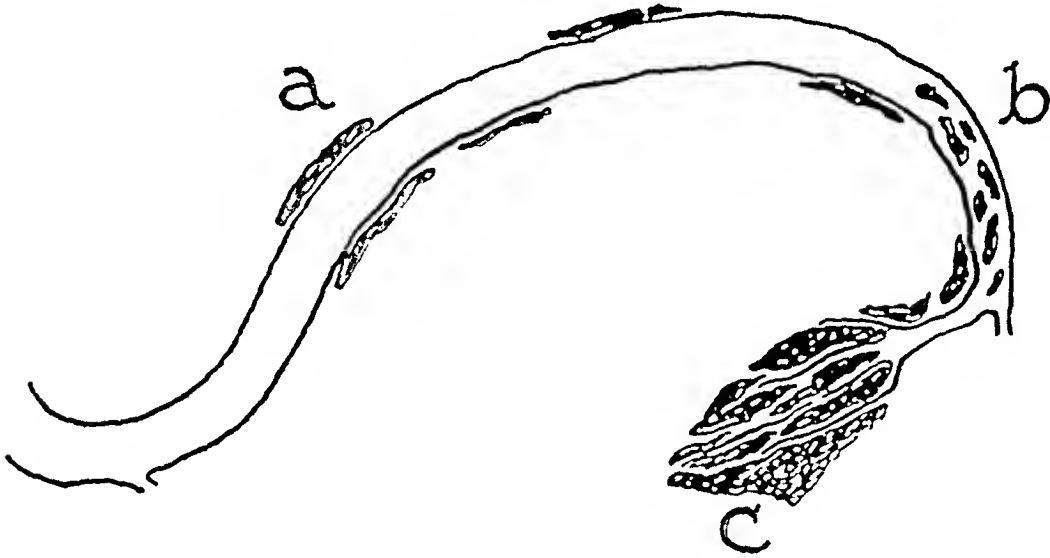


Fig. 2. Diagrammatic representation of the three types of relationship between nerves and Leydig cells. At 'a' are shown elongated groups of Leydig cells situated alongside the larger nerves running in the deep layers of the albuginea, at 'b' the Leydig cells are dispersed inside the nerve, and at 'c' the nerve fibres are interpenetrating intertubular masses of Leydig cells.

Fig. 1). As a nerve enters such a region alongside an interlobular artery of considerable size, it sinks into the loose collagenous tissue of the septulum. Directly larger masses of Leydig cells are reached, the nerve splits up into fascicles which interweave the cell-clusters. In this way a very intense blending of nerve fibres and Leydig cells is achieved. This is really an example of parenchymatous nerve termination. The obvious remaining problem is to find out whether a contact exists between neurofibrils and the elements of the Leydig tissue.

The ultimate connexions between nerves and Leydig cells

We have stated that intertubular masses of Leydig cells may be criss-crossed by nerve fibres. The question arises whether these fibres simply pass by the cells or whether they form a nervous contact with individual cells. We have been able to demonstrate that the latter possibility occurs.

When a sufficiently large number of preparations is examined it is

always possible to select sections where the entire terminal portion of one or more neurofibrils is revealed without interruption. In such cases the course of a neurofibril may be followed from the point where it was incorporated in a tiny nerve to the point where it ends by touching a Leydig cell. We attach great importance to these observations because they give



FIG. 3. Diagram from testis No. 4270 (section 10 of complete series) from sexually abnormal subject (aet. 37). In a region exactly corresponding to those photographed in Figs. 4-8, Plates I-III, a nerve divides around a group of Leydig cells. One fascicle proceeds into the cell cluster and examination of this field under high-power reveals some neurofibrils ending in tiny loops on the surface of Leydig cells.

direct evidence in a field where serious causes of error exist. Not all argyrophile fibrils are neurofibrils. The argyrophile reticulum in which the Leydig cells are lodged differs in several respects from neurofibrils. Considerable uncertainty would be felt, however, as to the precise nature of fragments of silver impregnated fibrils near Leydig cells if we had not worked with complete series and if we had not been able to obtain this direct evidence of fibrillary continuity from nerve bundles to the surface of Leydig cells (Fig. 3).

The impregnated neurofibrils stop at the surface membrane of the Leydig cells; they do not penetrate into the cytoplasm. Frequently these nerve-endings have the shape of a minute knob or even a hook, and sometimes this tiny end-organ seems to be surrounded by a homogenous

layer of a non-stainable material. This description covers chiefly the parenchymatous neuro-cellular connexions which we have classified as type *c*. But it should be noted that a similar contact of neurofibrils and Leydig cells has been observed in the type *b* relationship as well.

We might add that the blending of nerves and Leydig cells has only been observed in the testis after puberty. We have studied material from the 9-12 and the 12-15 years age-groups, and we have found that unless spermatogenesis has commenced, no intimate relationship between nerves and Leydig cells inside the parenchyma can be demonstrated. In foetuses (7-8 months old), and in the new-born, hilum cells have been found once or twice; their relationship with the nerves from the plexus deferentialis is definitely of a para- or peri-neural type.

Discussion

Up to the present the testicular nerves have been regarded as either vasomotor or sensory. Our observations show that the larger nerves, until they reach the parenchyma of the gland, pursue a peri-arterial course. It is, therefore, highly probable that vasomotor fibres are given off into the arterial wall. These vasomotor fibres, however, cannot be very conspicuous or numerous since we have not yet been able to follow their course histologically and they must be less in number than the fibres entering the intertubular masses of Leydig cells. We have not so far paid any attention to the question of the sensory nerves, but they must be relatively numerous considering the high sensitivity of the testis as an organ.

We have, however, demonstrated the intimate connexion between fibrils of the peri-arterial nerves and the intertubular groups of Leydig cells. We have further demonstrated that there may be three types of connexion and that the smallest fibrils possess small end-organs which actually touch the cells. We believe that these nerves must have some functional significance and suggest that their function may be secretory. This would be in line with recent evidence suggesting that a special area of the hypothalamus is intimately concerned with the whole complex of the sexual processes. The conception that the testicular nerves take part in such a neuro-humoral mechanism naturally suggests itself.

In this connexion we may mention recent work on the peculiar cells of the ovarian hilum. These cells may also enter into close relationship with the ovarian nerves and even present an intraneural distribution. We have already referred to the paper by de Amaral [1938], on reading which we were at once struck by the obvious analogy between the 'sympathico-tropic' cells of Berger [1922, 1932] situated in the ovarian hilum and the nerve connexions we have described in the fibrous coat of the testis. Berger

[1922] was the first worker to study these peri- or intra-neural cells of the hilum, which had previously been regarded as pheochrome elements belonging to the paraganglionic system. Berger, however, definitely excluded them from this system. At the time considerable discussion on this point arose between Berger [1932] and de Winiwater [1924] and their respective followers. We, however, agree with do Amaral that such subtle distinctions as between the significance to be attached to different shades in chromo-, argyro- and sidero-phil staining, which were put forward as arguments at the time, have no value from the histochemical point of view. The really significant point was that Berger was able to demonstrate that similar elements, also situated near the nerves, were to be found in the testes. He believes that the hilum cells originate from the Schwann syncytium and that they possess a 'neurocrine' function in Masson's sense of the word. Berger's observations have been, in the main, confirmed by Brannan [1927], Kohn [1928] & do Amaral [1938], though it should be mentioned that the last author was unable to demonstrate neurofibrils either in or around these cells. Although the latter author was chiefly concerned with the sympathiotropic or Berger cells of the ovarian hilum, he advanced the hypothesis that these cells are homologous with the interstitial cells of the testis and are 'the morphological substratum of the secretion of masculinizing hormones in women'

In our preliminary report [Sand & Okkels, 1938] on the intraneural Leydig cells we were uncertain as to the exact nature of the peculiar large neurotropic cells which we had observed inside the mediastinum testis. Continued work on this subject and the examination in serial section of the large quantity of material at our disposal has enabled us to generalize the problem. We believe that the elements which, outside the testicular parenchyma, are found beside or inside the nerves, are of the same nature as the massively grouped intertubular elements among which the terminal nerve fibres can be demonstrated; they are all morphologically and histochemically Leydig cells. Whether the functional significance of this intimate relationship between nerves and Leydig cells is of a secretory nature or whether a special phenomenon such as the 'neurocrinie' postulated by Masson is involved cannot be determined at present. The possibility exists of a biological mechanism by which the neurotropic Leydig cells may influence the testicular nerves by means of a special secretion. The nerves could thus transmit centripetal stimuli which might explain the psycho-sexual phenomena associated with the influence of the sex hormones on the central nervous system.

SUMMARY

1. The nerves of the human testis are far more numerous than has hitherto been assumed.

2. A great number of these nerves make contact with Leydig cells. Near the hilum these cells may occupy a perineural situation. In the deeper layers of the tunica albuginea intraneural Leydig cells may be found. Inside the parenchyma nerve fibres may interpenetrate groups of Leydig cells.

3. Neurofibrils may end by touching individual Leydig cells.

4. The intimate relationship between nerves and Leydig cells seems to be established at puberty.

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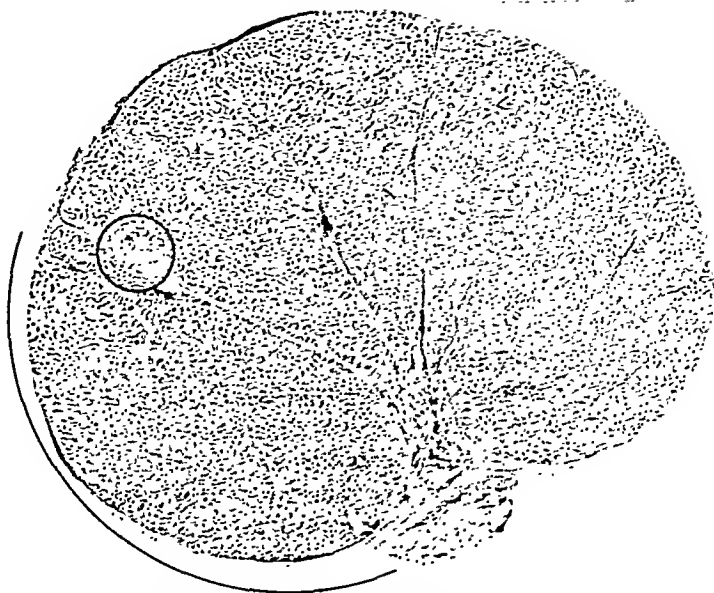


FIG. 1. Standard section of testis in the transverse plane. The curved line indicates the course of the periaarterial nerves which run around the testis deep to the tunica albuginea. These nerves enter successively peripheral parts of the septula testis. The circle indicates the region in which were found the nerves shown in Figs. 4-8 (Plates I-III).

FIGS. 4-8: Testis No. 4374; subject (act. 22) killed by traffic accident. The five figures are untouched microphotographs from corresponding regions of five sections picked from one complete series.

The photographed fields were located in a septulum about 3 mm. beneath the surface (cf. Fig. 1). The histological aspect of both testes was alike.

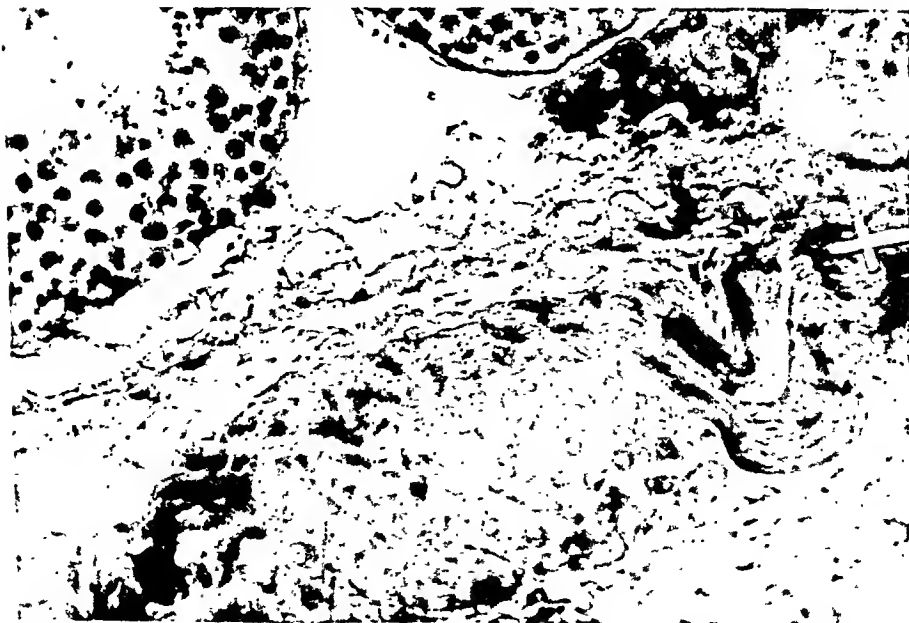


FIG. 4. Section No. 3 of the series. At the white cross is seen a medium-sized nerve which has reached a group of Leydig cells. Silver impregnation. $\times 400$.

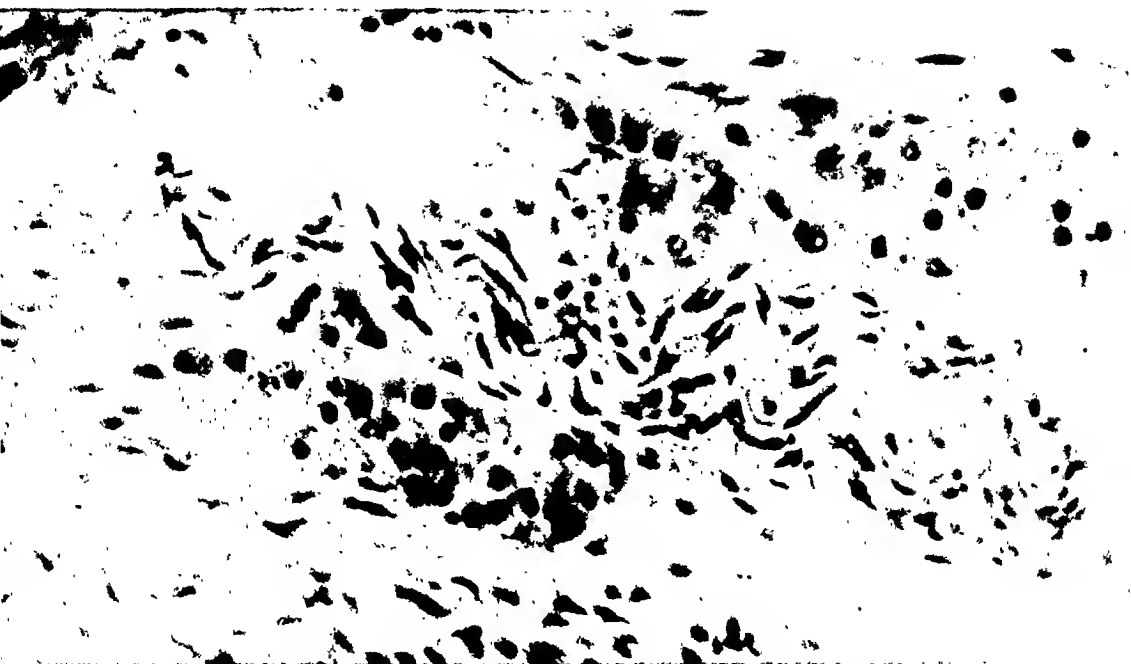
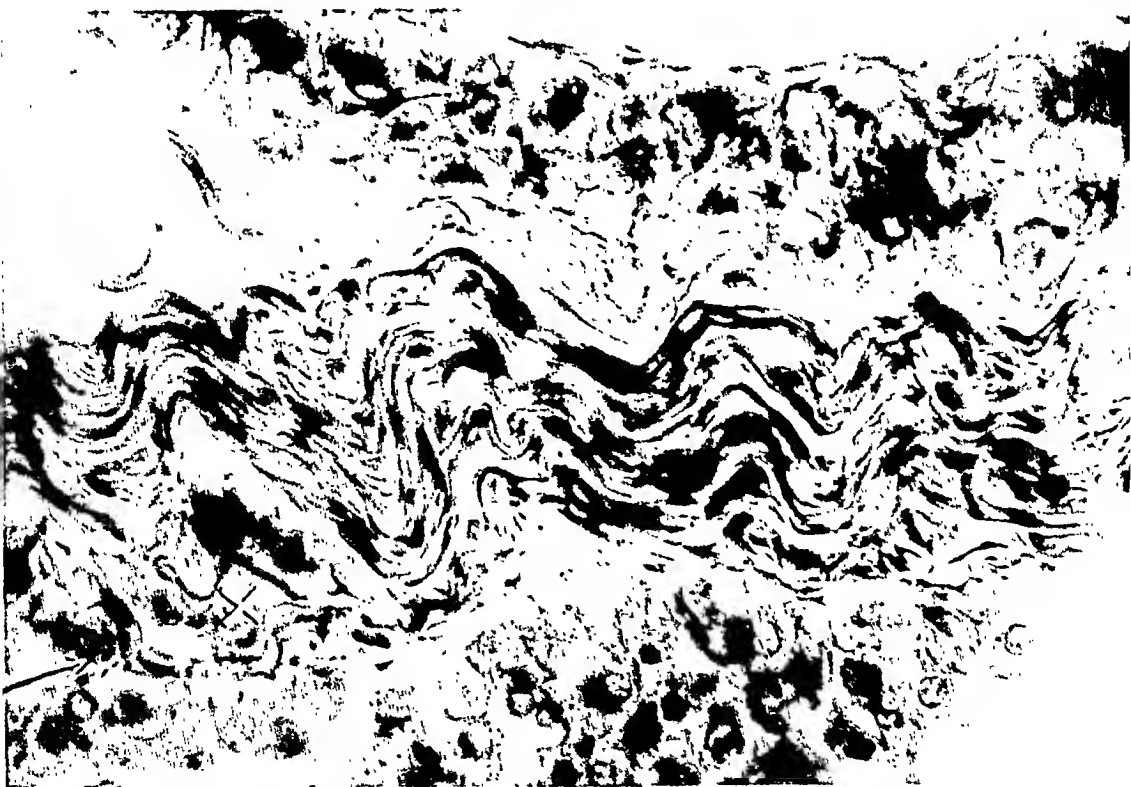


FIG. 5. Section No. 5 of the series. Another bundle of the same nerve passing between clusters of Le cells. Hoidenhain's iron haematoxylin stain. $\times 420$.



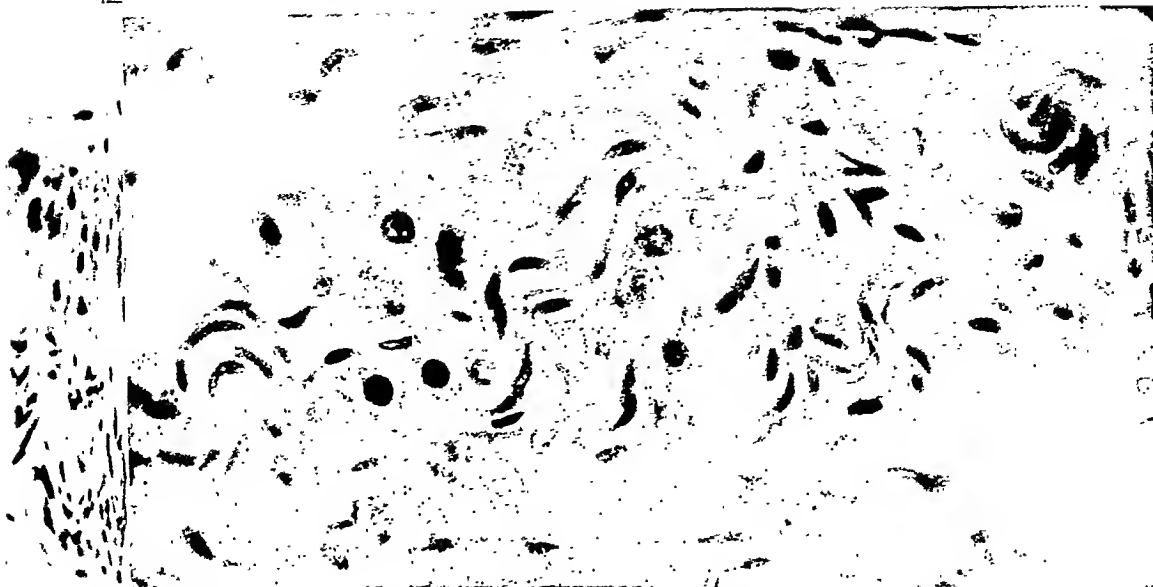


Fig. 7. Section No. 13 of the series. The finer ramifications of the nerve are here lost among the Leydig cells. Heidenhain's iron haematoxylin stain. $\times 510$.

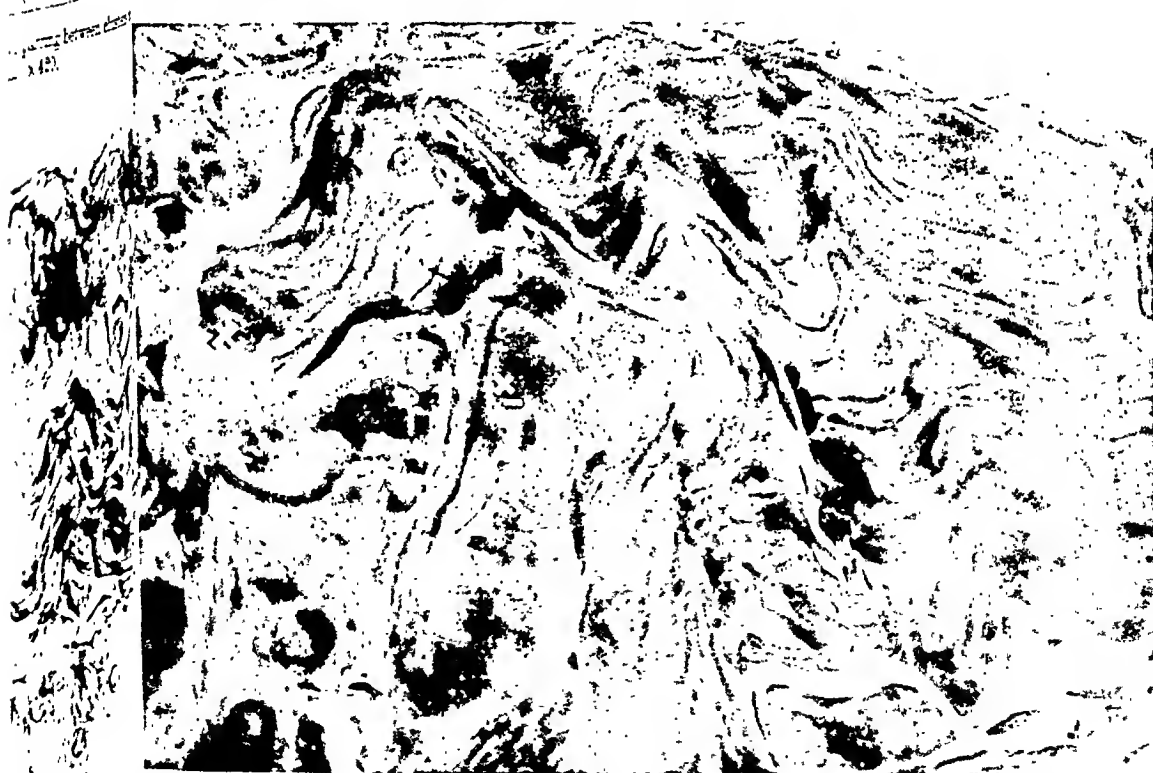


FIG. 8. Section No. 14 of the series (detail exactly corresponding to the field shown in Plate III, Fig. 7). A small fascicle is seen following its wavy course at \times . These fibres are seen in cross-section. Several single neurofibrils (\times) pass on to individual Leydig cells. Silver impregnation. $\times 600$.

Fig. 5. The white nerve
Silver impregnation. $\times 600$

SEXUAL DEVELOPMENT OF FOWLS DERIVED FROM EGGS TREATED WITH OESTRADIOL BENZOATE

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(Received 4 December 1939)

If hens' eggs are treated during the first week of incubation with oestrogen, the male chicks may show signs of feminization [Willier, Gallagher & Koch, 1935, 1937; Dantchakoff, 1938; Wolff & Ginglinger, 1935 *a, b*; Ludwig & von Ries, 1936, 1937]. An ovary or ovotestis is found in place of the left testis, the right gonad remains more or less rudimentary, while the Mullerian ducts show none of the usual atrophy. The question arises how these hormonally produced sex organs develop during post-natal life. Dantchakoff [1938] allowed a few of these feminized chicks to grow up and found a gradual regression towards the male, both in the gonads and the secondary characters. Ludwig & von Ries, however, observed no such regression.

In the present series of experiments 159 chicks derived from eggs treated with oestradiol benzoate were killed at varying times after hatching to give more detailed information about this question.

MATERIALS AND METHODS

Animals and treatment. 477 White Leghorn eggs were placed in an incubator and 400 of them were each injected with 300 μ g. (3,000 I.B.U.) of oestradiol benzoate in 0.1 ml. oil on the second day. The remaining 77 eggs were left untreated to serve as controls. 159 of the treated eggs hatched and 50 of the controls.

The chicks, both experimental and control, were separated into three groups, one group being killed a week after hatching, the second group two months after hatching and the third group after nine months.

There was considerable spontaneous mortality during the experimental period in spite of precautions taken to prevent the outbreak of infection. It is possible that the treatment of the eggs caused a general weakness favouring the occurrence of disease, though the mortality did not particularly affect the feminized male chicks. The control birds also showed a high death-rate, though this may have been due to the fact that they were not segregated from the experimental animals and may have been infected from the latter. Those chicks which died during the week pre-

ceding any of the autopsy days were included in the corresponding groups. Data are not included from those animals dying in the intermediate periods since no additional information was derived from them.

Examination at autopsy

Macroscopic aspect of the gonads. In new-born chicks it is possible approximately to determine the sex by macroscopic examination of the gonads. The right gonad is only rudimentary in the female, while in the male it is the same size as the left one. Intersex chicks therefore have right gonads 25-66% of the size of the left gonads.

Microscopic aspect of the gonads. The histological examination of the gonads was chiefly carried out on the left gonad since this is present in both sexes and the presence of both ovarian and testicular tissue in this gland would be conclusive proof of intersexuality.

Other sex characters. The birds were also examined for the development of the Mullerian ducts, Wolffian ducts and secondary sexual characters such as the combs, wattles and feathering.

The results are summarized in Table I.

EXPERIMENTAL

Group I (killed one week after hatching)

This group consisted of 42 treated animals and 13 controls. The results are not presented in detail since they do not differ from those previously reported.

Macroscopic aspect of the gonads. A definite sex reversal was observed since, of the treated birds, 33 were female, 8 were intersex and only 1 was male. The somewhat abnormal sex relation found in the 13 control birds (9 females to 4 males) may be regarded as occurring by chance.

Histological examination of the gonads. The embryonic gonad consists of a cortex and a medulla. The cortex contains groups of cells which, after hatching, develop into follicles. The medulla gradually atrophies. The testis originates in a tissue corresponding to the ovarian medulla.

The left gonads of the female and intersex chicks derived from the treated eggs consisted entirely of ovarian tissue, demonstrating the potent feminizing action of oestradiol benzoate. The right gonads of the intersex birds were composed of poorly developed testicular tissue. In the single treated male bird two normal testes were present.

Development of the Mullerian duct. A left Mullerian duct was present in all the treated chicks with the exception of the single male animal. This proves that oestradiol benzoate directly or indirectly arrests the physiological atrophy of the organ in genetic males.

The right Mullerian duct, which at birth is normally absent in the male

and only a few mm. in length in the female (as confirmed without exception in the control birds), was found to be still existent in 41-42 cases. The length of the right duct in 25 chicks was greater than in the normal untreated female, the lengths of the right and left ducts being, in some cases, equal. Practically all the ducts were wholly or partially swollen, containing a clear fluid in the lumen.

Development of the Wolffian ducts. No abnormalities were observed.

Table I

	No. of birds	Sex relation*	Mullerian ducts present	Histological examination of left gonads			
				No. examined	No. of testes	No. of ovotestes	No. of ovaries
<i>Experimental birds</i>							
Group I	42 (4)†	1 male	0	1	1	—	—
		33 female	33	27	—	—	27
		8 intersex	8	5	—	—	5
Group II	41(19)	0 male	—	—	—	—	—
		19 female	19	18	—	—	18
		22 intersex	21	19	14	5	—
Group III	27 (0)	17 male	12	16	14	2	—
		10 female	10	5	—	—	5
		0 intersex	—	—	—	—	—
<i>Control birds</i>							
Group I	14 (1)	4 male	0	3	3	—	—
		9 female	9	6	—	—	6
		0 intersex	—	—	—	—	—
Group II	16 (6)	8 male	0	7	7	—	—
		8 female	8	7	—	—	7
		0 intersex	—	—	—	—	—
Group III	0 (—)	— male	—	—	—	—	—
		— female	—	—	—	—	—
		— intersex	—	—	—	—	—

* determined by macroscopic examination.

† the numbers in parentheses represent the birds which died during the week preceding the day of autopsy and which were included in the corresponding group.

Group II (killed two months after hatching)

This group consisted of 41 treated birds and 16 untreated birds.

Macroscopic aspect of the gonads. The sex of these birds was determined by the already described macroscopic method. The treated animals proved to consist of 19 females and 22 intersexes. In two months, therefore, conditions had changed importantly, the percentage of females having dropped to about normal and the number of intersexes showing a commensurate increase. Evidently the surplus of females present at hatching

was mutated into intersexes. The left gonads of the intersexes in appearance resembled ovaries, although invariably smaller, less flattened and not showing the characteristic lobulated structure. The right gonads rather differed in size, in a few cases having attained the size and the aspect of completely developed testes. Signs of a partial return to the male sex therefore are not limited to the left gonad but also manifest themselves in the right gonad.

Histological examination of the gonads. The treated chickens macroscopically denoted as females all possessed an ovary on the left side, but the left gonads of the macroscopic intersexes were found to contain testis tissue in all cases, although the latter tissue, so far as the number of tubules per unit surface is concerned, showed appreciably poorer development than in the control chickens. In 5 of the 19 left gonads of the intersex chicks examined, beside the testis tissue, one or more well-developed ovarian follicles were found, situated at the periphery of the organ. These gonads therefore proved to be ovotestes (see Plate I, Fig. 1). The number of follicles found in the ovotestes was in no case considerable, being 6 in the largest transverse section; a cross-section of a normal ovary shows a much greater number of follicles. Some of the left gonads revealed remnants of cortex, such as are found in the ovary of the newly born chick.

The right gonads of the intersex chicks all consisted of testis tissue, which, however, as in the case of the left gonad, showed less complete development than the testes of the controls.

Since about 75% of the chickens macroscopically denoted as females possessed gonads containing only testis tissue, they proved to be males. The method for determining the sex by macroscopic examination of the gonads is therefore not reliable when applied to two-months-old chickens.

Development of Mullerian ducts (oviducts). The abnormalities of the Mullerian ducts present at hatching (swelling, abnormal length of the right duct, &c.) were no longer found in the treated female chickens, whereas for the intersex birds they had, on the contrary, increased. In the females the right Mullerian duct, except in two cases, had disappeared. This disappearance is a physiological process, which was evidently retarded by the administration of oestradiol benzoate to the embryos. The intersex birds in most cases still possessed two well-developed Mullerian ducts, the right one being in many cases of abnormal length. Both ducts in most cases were enormously swollen, the circumference being often considerably greater than that of the cloaca: badly swollen parts alternated with entirely normal parts. In some places the cystic vesicles thus formed had grown together with the surrounding organs (see Plate I, Fig. 2). The wall of the ducts was thin and transparent; histologically it did not show anything noteworthy, though giving the impression of being

considerably stretched. In some instances the lumen of the duct was filled wholly or partially with a massive yellow-white substance. This substance was found histologically to consist entirely of leucocytes.

Development of Wolffian ducts (ducti deferentes). Unfortunately the condition of the Wolffian ducts of the birds of this group was not investigated in detail. The general impression, however, was that no important abnormality was present.

Group III (killed nine months after hatching)

From the second month onward, an indication of the sexual development of the chicks could be obtained by inspecting the external sexual characters (comb, feathering, &c.). An inspection of the birds when they were four months old revealed the following facts:

Of the 42 birds still alive at that time, 15 were like entirely normal females. 25 others showed the male secondary characters, the comb, however, in every instance being smaller than the comb of the 3 control males. The feathering of the treated males was midway between the male and the female type. This need not indicate the presence of intersexuality, since the immature type of feathering is identical with the female one and therefore a transitory period exists, during which both male and female feathers are found. The feathering of the control males was much closer to the male type, so that the mixed feathering of the treated males indicated that either sexual underdevelopment or intersexuality was present. In 2 treated animals sex could not be established by external examination.

On the last day of autopsy 27 birds were still alive; 10 of them were regarded as normal females and 17 as normal males except for a somewhat small comb. The feathering in no case showed appreciable abnormalities. Autopsy revealed the following facts:

Macroscopic aspect of the gonads. From the appearance of the gonads it was easy to establish whether the organ concerned was a testis or an ovary. Sex relation, determined in this way, fully corresponded with the relation established by external inspection. The left testis in some instances showed a slightly lobulated structure, suggesting the lobulated structure of the immature ovary. The testis appeared smaller than normal, a true appreciation, however, being difficult, since in male fowls the size of the testis varies considerably. In 4 cases the right testis was considerably smaller than the left one, being similar to the rudimentary left gonad in females.

Histological examination of the gonads. The ovaries showed no abnormality. The organs macroscopically denoted as testes contained, without exception, testicular tissue. Though comparison with the testes of control

birds was not possible, since the latter had all died, the size of the tubules in most testes appeared to be normal. Many tubules contained spermatozoa. Three animals possessed testes which without doubt showed poor development, the diameter of the tubules being much smaller and no spermatozoa being present. At the periphery of the left gonad in 2 males, beside the (in these cases well-developed) testicular tissue, a small number of ovarian follicles was found. Even nine months after the treatment, therefore, this important sign showing the effect of oestradiol benzoate was still present.

Development of Mullerian ducts (oviducts). The oviducts of the females appeared entirely normal, some of them containing eggs. Twelve of the 17 males still possessed Mullerian ducts, in most instances on both sides. These organs showed the same abnormalities as the Mullerian ducts of the intersex birds of group II. They were enormously swollen, contained fluid and in several cases were transformed into large purulent masses. The relation between the size of the bird and the size of the ducts was about the same as in the two-months-old chicken, so the duct had grown considerably. No resemblance existed between the well-shaped oviducts of the females and the degenerative products in the males.

Development of Wolffian ducts (ducti deferentes). The ducti deferentes in many cases showed under-development, they were thinner than normal; the spiral structure in some instances was not present and sometimes the duct was hardly visible.

DISCUSSION

From these experiments it is obvious that the male embryos, which by administration of oestradiol benzoate to the eggs become female or intersex, show a gradual regression to their genetic (male) sex during post-natal life. The main part of this process has already taken place during the first two months after hatching. One is inclined to ask why the hormone-produced ovary, though by no means showing a visible difference from the genetic one, is unable to develop as a normal ovary. The difference between the genetic and the hormone-produced female probably lies in the chromosomal picture, or, according to Goldschmidt [1931], in the relation between the male and female substances, (F) and (M). The formation of follicles in the cortex of the fowl ovary usually begins a few days after birth. A suitable hypothesis would be that the male chromosomal picture, or the male relation F/M, is not in accordance with that prevailing during the normal formation of this typical ovarian tissue. This, however, turned out to be unsatisfactory, since in the left gonad of the feminized male a few follicles do develop, some of them still existing nine months after hatching.

Another, and very simple, explanation for the poor development of the

hormone-produced ovary would be that this development is arrested by the appearance of testis tubules in the medulla. This raises the question how testis tubules can originate in an apparently normal ovary, the probable explanation being that the normal stimulus for this process (in males), which was for a time suppressed by oestradiol benzoate, again becomes manifest.

In previous papers some hypotheses regarding the absence of testicular tissue in the left gonad of the feminized male embryo were discussed. Thus Gaarenstroom [1937, 1938, 1939] claims that a certain action of the embryonal hypophysis is necessary for the formation of testis tissue, hypophyseal activity being suppressed by oestradiol benzoate. Or oestradiol benzoate may be able to prevent the formation of the inductive substance by which the testis is originated. Since inductive substances (according to Needham [1937]) are, like oestradiol benzoate, steroids, it is by no means unlikely that the abundant presence of one substance arrests the formation of the other one.¹ Dantchakoff [1938] suggests that the sufficient migration of primordial sex cells into the medulla of the indifferent gonad must precede the development of testis tubules and that this migration is arrested by oestradiol benzoate. According to these hypotheses, the origin of testis tissue is explained either by an activity of the hypophysis, by the formation of an inductive substance or by a migration of primordial sex cells. The occurrence of such processes should then be possible after birth. The above-mentioned hypotheses, however, are not at present supported by much evidence, so that to analyse the consequences of them would be of little value.

The question may arise, if by repeated administration of oestradiol benzoate during embryonal and post-natal life the maintenance of the hormone-produced ovary would be facilitated. We tried the repeated administration to a group of 25 eggs; the damage done by the treatment, however, caused the death of all the embryos. Dantchakoff [1938] treated a few chickens, considered as hormonal females, after birth with oestrogens, but in spite of the treatment the birds changed into males.

Another effect to which attention is drawn is the peculiar development of the Mullerian duct in the genetic males. When, instead of a left testis, an ovary is originated, the abnormal maintenance of the Mullerian ducts is not surprising. When, however, the ovary is transformed into a testis, one would expect the atrophy of the Mullerian ducts to occur. Instead, an enormous swelling of the ducts takes place. Some unknown factor evidently causes a strong secretory activity of the epithelium of the duct.

The presence in some of the nine-months-old fowls of under-developed testes, small combs and poorly developed Wolffian ducts indicates

¹ I am indebted to Dr. Freud for the suggestion of this hypothesis.

that testicular development, even at this age, is still retarded. The delayed formation of the testis is evidently able to disturb for a very long time the development of this organ.

SUMMARY

As reported by several authors, male chickens at hatching show signs of feminization if during embryonal life they are treated with oestrogens. To study the post-natal development of the sex organs of these feminized males, a number of chickens, treated during embryonal life with oestradiol benzoate, were killed at various periods after hatching. Of 159 treated and 50 non-treated birds, group I was sacrificed one week after hatching, group II two months after hatching and group III nine months after hatching.

Group I. The treated birds showed signs of a strong feminization. The sex-ratio was moved in the female direction, ovaries and Mullerian ducts being present in at least 80% of the chicks.

Group II. Of the treated birds about 50% were normal females. The other 50% possessed gonads, containing in every instance testicular tissue, some of them also containing some well-developed ovarian follicles. These birds, though apparently genetic males, still possessed Mullerian ducts. The ducts in most cases were enormously swollen; the Mullerian ducts of the females, on the other hand, were entirely normal.

Group III. The sex-ratio of the treated birds, which at this age can easily be established by external inspection, showed no abnormality. The treated males proved to possess normal testes in most cases; in two testes a few small follicles were observed. Mullerian ducts of abnormal character were still found in the majority of the males.

As indicated by these experiments, the feminized males could not maintain their female sex organs during post-natal life. The hormone-produced ovaries were transformed into testes, ovarian remnants being still present in only a few cases. The Mullerian ducts of the feminized males, though showing abnormal development, did not, however, atrophy.

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FIG. 1. Left gonad of an intersex chicken, eight weeks after hatching. Many testicular tubules are present, but one well-developed ovarian follicle still remains.

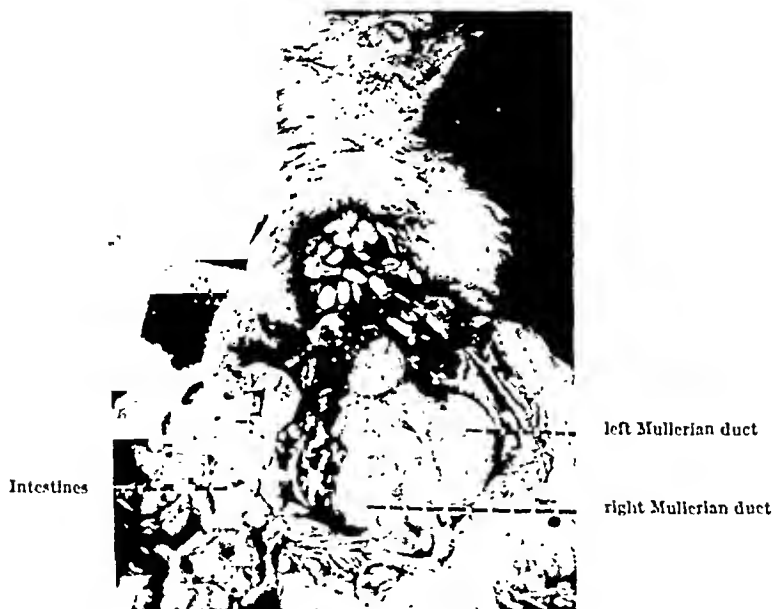


FIG. 2. Enormously swollen Mullerian ducts of an intersex chicken, eight weeks after hatching.

THE METABOLISM OF THE PARENT COMPOUNDS OF SOME OF THE SIMPLER SYNTHETIC OESTROGENIC HYDROCARBONS

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(Received 20 December 1939)

EXPERIMENTS on the recovery of synthetic oestrogenic phenols, related to stilbene and diphenylethane [Dodds, Golberg, Lawson & Robinson, 1939], from the urine of female rabbits, have shown that the recoveries are very high compared with that of oestrone under the same conditions [Stroud 1939]. 4:4'-Dihydroxy- γ : δ -diphenyl-*n*-hexane [Campbell, Dodds & Lawson, 1938] gave a recovery of 21.3%, 4:4'-dihydroxy- α : β -diethylstilbene and 4:4'-dihydroxy- γ : δ -diphenyl- β : δ -hexadiene [Dodds *et al.* 1939], recoveries of 25.2% and 7.2% respectively, compared with 1.5% found for oestrone under the same conditions. From these figures it is apparent that the synthetic oestrogenic phenols are considerably more stable in the body than oestrone and that their metabolic paths differ widely from that of the naturally occurring hormone.

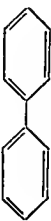

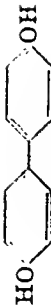




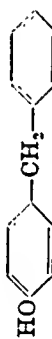
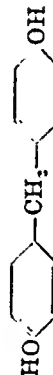



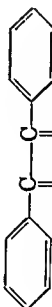
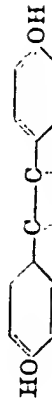
In studying the metabolism of these compounds it was considered of importance to include the oestrogenic hydrocarbons and also other hydrocarbons which, whilst themselves having no oestrogenic activity, have corresponding phenolic compounds showing oestrogenic activity. For the purpose of the experiment diphenyl ether is included in the hydrocarbon series. Table I shows the compounds used in the experiments with their corresponding mono- and di-hydroxy compounds, together with their oestrogenic activities. The unit of oestrogenic activity adopted is the smallest weight of the compound which will produce full oestrous response when injected into ovariectomized rats, and when a substance is stated to be inactive it infers that no oestrous response was obtained with an injection of 100 mg.

METHODS

In each experiment 3 grammes of the compound under consideration were dissolved in 80 ml. sesame oil and two mature non-pregnant female rabbits were each injected with 2 ml. of this solution daily for a period of 20 days, the urine being collected over this period and also for a further 7 days after the last injection. Before the commencement of the injections

¹ Working on a grant from the Medical Research Council.

Table I

Parent compound	Mono-phenol	Di-phenol
		
Diphenyl (Inactive)	(Inactive)	(100 mg.)
		
Diphenyl ether (Inactive)	(100 mg.)	(100 mg.)
		
Diphenylmethane (Inactive)	(Inactive)	(50 mg.)
		
Stilbene (25 mg.)	(5 mg.)	(5 mg.)
	Not known	
Diphenyl-hexadiene (10 mg.)		(0-4 μ g.)

of the compound, control urine was collected from the rabbits which had received a daily injection of 2 ml. sesame oil. The urine was stored in a refrigerator as soon as possible after being passed, toluene being added as a preservative.

The urine was extracted with benzene in a continuous extractor for 20 hours. This extract containing the material extractable before hydrolysis was termed the 'free' extract. The extracted urine was then acidified with concentrated hydrochloric acid (50 ml. acid per litre of urine) boiled under reflux for 2 hours, and again extracted with benzene for 20 hours to give the 'combined' extract.

Each benzene extract, 'free' and 'combined', was evaporated to a convenient volume (approximately 700 ml.), extracted 3 times with 75 ml. portions of saturated sodium bicarbonate solution to remove acids, 5 times with 100 ml. portions of 2N sodium hydroxide solution to remove phenols, and finally washed twice with 50 ml. portions of water, the water washings being added to the sodium hydroxide washings. The benzene solution was then evaporated to dryness to give the neutral fraction of the extract. The sodium bicarbonate washings were acidified to litmus with hydrochloric acid and extracted twice with 75 ml. portions of ether. The ether solution was washed twice with water, dried over anhydrous sodium sulphate and evaporated to dryness to give the acid fraction of the extract. The sodium hydroxide washings were acidified to litmus with hydrochloric acid and extracted with ether as before. The ether solution was washed twice with 50 ml. portions of water, dried over sodium sulphate and evaporated to dryness, to give the phenolic fraction.

Where biological assay was carried out on the phenolic fractions, the fraction was dissolved in acetone and made up to a definite volume. Aliquot portions of this solution were pipetted into test-tubes and sufficient acetone was added to make the volume up to 0.5 ml., 17.5 ml. sesame oil were then added and the whole well shaken. For each test 5 ovaricetomized female rats were used, each rat being injected twice daily with 0.5 ml. of the oil solution for 3 days, vaginal smears being taken on the 3rd, 4th and 5th days. The unit adopted was the lowest dose that would give 100% oestrous response under these conditions.

RESULTS

Diphenyl.

Weight of phenolic fraction obtained from the 'free' extract = 1.166 g.

Weight of phenolic fraction obtained from the 'combined' extract

= 1.317 g.

The phenolic fraction of the 'free' extract was dissolved in acetone and poured into water to precipitate the phenol. The phenol was filtered

off, 594 mg. of crystals, m.p. $162^{\circ}\text{C}.$, being obtained. After recrystallization from benzene these melted at $163-4^{\circ}\text{C}.$ and did not depress the melting-point of 4-hydroxydiphenyl (m.p. $164^{\circ}\text{C}.$).

The phenolic fraction of the 'combined' extract was treated in the same way as above, 169 mg. of 4-hydroxydiphenyl being obtained.

No diphenyl was recovered from the urine.

Diphenyl ether.

Weight of phenolic fraction obtained from the 'free' extract = 1.345 g.

Weight of phenolic fraction obtained from the 'combined' extract
= 0.467 g.

The phenolic fraction of the 'free' extract was extracted five times with boiling water, the solution being filtered hot. On cooling, 620 mg. of crystals, m.p. $82-3^{\circ}\text{C}.$, were obtained. After recrystallization from petroleum ether these melted at $84^{\circ}\text{C}.$ and did not depress the melting-point of 4-hydroxydiphenyl ether (m.p. $84^{\circ}\text{C}.$).

The phenolic fraction of the 'combined' extract was treated in the same way as above, 47 mg. of 4-hydroxydiphenyl ether being obtained.

No diphenyl ether was recovered from the urine.

Diphenylmethane.

Weight of phenolic fraction obtained from the 'free' extract = 0.964 g.

Weight of phenolic fraction obtained from the 'combined' extract
= 0.518 g.

The phenolic fraction of the 'free' extract was extracted five times with boiling water, the solution being filtered hot. On cooling, 394 mg. of crystals, m.p. $82^{\circ}\text{C}.$, were obtained. On recrystallization from petroleum ether these crystals melted at $84^{\circ}\text{C}.$ and did not depress the melting-point of 4-hydroxydiphenylmethane (m.p. $84^{\circ}\text{C}.$).

The phenolic fraction of the 'combined' extract was treated as above, 48 mg. of 4-hydroxydiphenylmethane being obtained.

No diphenylmethane was recovered from the urine.

Stilbene.

Weight of phenolic fraction obtained from the 'free' extract = 0.579 g.

Weight of phenolic fraction obtained from the 'combined' extract
= 0.542 g.

The phenolic fraction of the 'free' extract was dissolved in a little hot glacial acetic acid and allowed to cool. 42 mg. of crystals, m.p. $274^{\circ}\text{C}.$, were obtained. On further concentration the acetic acid solution yielded a further 12 mg. After recrystallization from acetic acid these crystals melted at $278^{\circ}\text{C}.$ and did not depress the melting-point of 4:4'-dihydroxystilbene (m.p. $280^{\circ}\text{C}.$).

The phenolic fraction of the combined extract was treated in the same way as above, 33 mg. of 4:4'-dihydroxystilbene being obtained.

Weight of acid fraction obtained from the 'combined' extract = 4.082 g.

Weight of acid fraction obtained from the control 'combined' extract
= 2.413 g.

The acid fraction of the 'combined' extract was extracted five times with petroleum ether, the resulting solution then evaporated to dryness. This dry extract was dissolved in boiling water, the solution filtered and allowed to cool. 2.551 g. of a crystalline acid, m.p. 117° C., were obtained. After recrystallization from benzene the crystals melted at 120° C. and did not depress the melting-point of benzoic acid (120–1° C.).

The acid fraction from the control 'combined' extract was treated as above, 1.161 g. of benzoic acid being obtained.

Thus 1.39 g. benzoic acid can be directly attributed to the stilbene injected.

No stilbene was recovered from the urine.

Diphenyl-hexadiene.

Weight of phenolic fraction of the 'free' extract = 0.457 g.

Weight of phenolic fraction of the 'combined' extract = 0.439 g.

From these fractions no crystalline phenols were isolated. The fractions were dissolved in acetone and aliquot portions of the solution were dissolved in sesame oil and injected into ovariectomized rats. The phenolic fractions from the control urine were dealt with in the same way. From the biological assay it was seen that the phenolic fractions of the 'combined' extracts made from the experimental and control urines had approximately the same oestrogenic activity, but the phenolic fraction from the 'free' extract contained approximately 20 R.U. of oestrogenic activity more than the corresponding control fraction.

Weight of neutral fraction of free extract = 0.776 g.

This fraction was dissolved in a small amount of ether and the insoluble matter filtered off, washed with ether and dried. Yield 21 mg., m.p. 178° C. After recrystallization from acetone these crystals melted at 184° C.

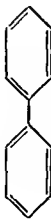
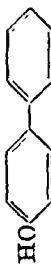
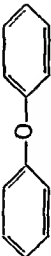
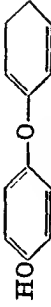





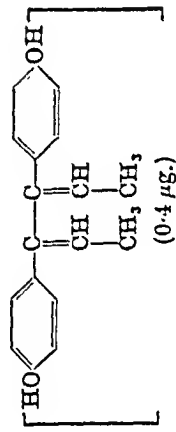
Analysis (Weiler and Strauss).

Found C = 80.14%	H = 6.64%	M.W. = 297
$C_{11}H_{10}O_2$ requires C = 81.2%	H = 6.80%	M.W. = 266
$C_{11}H_{10}O_2$ requires C = 79.9%	H = 6.72%	M.W. = 240

Owing to the small amount of material available these crystals have not yet been identified.

No diphenyl-hexadiene was recovered from the urine.

Table II. *Summarized results*

Parent compound	Phenolic metabolic product	Yield		
		'Free'	'Combined'	Total
 (Inactive)	 (Inactive)	594 mg. = 19.8%	169 mg. = 5.6%	763 mg. = 25.4%
 (Inactive)	 (100 mg.)	620 mg. = 20.7%	48 mg. = 1.6%	668 mg. = 22.8%
 (Inactive)	 (Inactive)	394 mg. = 13.1%	48 mg. = 1.6%	442 mg. = 14.7%
 (25 mg.)	 (5 mg.)	54 mg. = 1.8%	33 mg. = 1.1%	87 mg. = 2.9%
 (10 mg.)	 (0.4 μg.)	[20 r.u. = 8 μg.]	—	[8 μg.]

DISCUSSION

The first point of interest arising from these experiments is the fact that in no case was any trace of the original compound recovered from the urine.

From Table II it can be seen that in every case the injected compound has given rise to a phenolic metabolic product. Although no phenol was isolated in the pure state from the urine of the rabbits receiving diphenyl-hexadiene, the fact that the phenolic fraction of the 'free' extract shows greater oestrogenic activity than the control urine demonstrates the presence of an oestrogenic phenol, probably 4:4'-dihydroxy- γ : δ -diphenyl hexadiene, in minute quantities.

The yields of the phenols obtained give rise to many interesting considerations. From Table II it appears that the yields of phenols obtained vary inversely as to the oestrogenic activity of the parent compound injected and also inversely as to the oestrogenic activity of the phenol produced. Diphenyl and diphenylmethane, which are both inactive in a dose of 100 mg., give rise to 4-hydroxydiphenyl and 4-hydroxydiphenylmethane, both of which are inactive in a dose of 100 mg., in good yields. Diphenyl ether, which is also inactive in a dose of 100 mg., gives rise to 4-hydroxydiphenyl ether, which is active in a dose of 100 mg., also in a good yield. In this substance we have the case of an inactive parent compound being converted in the body to an active phenol. Stilbene, which is active in a dose of 25 mg., is converted in the body to 4:4'-dihydroxystilbene, the oestrogenic activity of which is five times as great as that of stilbene, being active in a dose of 5 mg., in a small yield. Diphenyl-hexadiene, active in a dose of 10 mg., is probably converted to the extremely active 4:4'-dihydroxy- γ : δ -diphenyl- β : δ -hexadiene, which is active in a dose of 0.4 μ g., but in this case the yield is minute. It is of interest to note that, of the compounds used in the experiment, stilbene was the only one to give rise to a diphenolic metabolic product.

Particular attention was paid to the acid fractions of the combined extracts in the experiments, but only in the case of stilbene could excess of benzoic acid above that found in the corresponding fraction of the control urine be found. 1.39 g. of benzoic acid were found attributable to the oxidation of stilbene, this amount representing 34.2% of the injected stilbene.

The identity of the neutral metabolic product isolated from the neutral fraction of the 'free' extract prepared in the diphenyl-hexadiene experiment has not yet been determined owing to the small amount of the compound available.

It is to be hoped that further work along these lines will furnish useful

information on the problem of the relationship between molecular structure and oestrogenic activity.

SUMMARY

The metabolism of diphenyl, diphenyl ether, diphenylmethane, stilbene and γ : δ -diphenyl- β : δ -hexadiene in normal female rabbits has been investigated.

From the urine of the rabbits receiving these substances none of the original substance injected was recovered.

All the compounds used gave rise to phenolic metabolic products. 4-hydroxydiphenyl was isolated from the urine of rabbits receiving diphenyl in a yield of 25.4% of the diphenyl injected; 4-hydroxydiphenyl ether from the urine of rabbits receiving diphenyl ether, yield 22.3%; 4-hydroxydiphenylmethane from the urine of rabbits receiving diphenylmethane, yield 14.7%; 4:4'-dihydroxystilbene from the urine of rabbits receiving stilbene, yield 2.9%; from the urine of rabbits receiving γ : δ -diphenyl- β : δ -hexadiene 20 rat units of a highly active oestrogenic phenol, probably 4:4'-dihydroxy- γ : δ -diphenyl- β : δ -hexadiene were obtained.

Stilbene is converted in the rabbit to benzoic acid, the amount isolated being equivalent to 34.2% of the stilbene injected.

I wish to thank Professor E. C. Dodds for his kind interest and suggestions made during the course of this work. I am also grateful to Mr. I. A. Hepple for performing the injections and to Mr. S. Graves for looking after the rabbits and making the urine collections.

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THE PRODUCTION OF OVULATION IN THE RABBIT BY THE INTRAVENOUS INJECTION OF SALTS OF COPPER AND CADMIUM

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THE augmentation of the action of various gonadotrophic preparations, usually of hypophyseal origin, by the addition of inert substances, has occupied the attention of many investigators. Various substances of animal or vegetable origin have been found to increase the potency of pituitary extracts, but zinc sulphate [Maxwell, 1934], various salts of copper, and, to a lesser extent, of iron [Fevold, Hisaw & Greep, 1936] are also effective. Fevold *et al.* [1936] further reported that salts of manganese, aluminium and calcium produced little or no augmentation. For a fuller bibliography and discussion of this question Deanesly [1939] may be consulted.

It is generally accepted that the action of zinc salts, and indeed of the majority of augmenting substances, is attributable to their decreasing the rate of absorption and thereby increasing the effectiveness of the active principles. However, Fevold *et al.* [1936] showed that the action of copper salts could not always be of this nature, as, in contradistinction to zinc salts, they failed to augment the action of F.S.H. in hypophysectomized rats, but produced ovulation when injected by themselves intravenously into rabbits. It was concluded that the activity of copper salts might be due to a catalytic action on the synergism supposed to exist between F.S.H. and L.H. Saunders & Cole [1938] confirmed the fact that intravenously injected copper salts will cause ovulation in mature female rabbits in doses of 25 mg., whereas zinc salts are ineffective in doses up to 50 mg.

Further results on the production of ovulation in rabbits by the injection of non-specific substances were published by Marshall, Verney & Vogt [1939], who showed that the intravenous injection of picrotoxin in doses which were lethal in 20% of cases, and convulsive in 75%, often caused ovulation, or the appearance of enlarged, cystic, or haemorrhagic follicles. Several other drugs, mostly stimulants of the central nervous system, were ineffective. Their previous study of the production of ovulation by electrical stimulation of the central nervous system [Marshall & Verney, 1936] led these authors to postulate that picrotoxin has a selective

stimulating effect on the anterior lobe of the pituitary body, causing it to liberate or produce gonadotrophic material. A similar explanation may well apply to the action of copper salts. It has been a regular observation that ovulation produced by pharmacological means tends to occur much later than that following normal coitus or the injection of gonadotrophic extracts. Thus, ovulation occurs within 12 hours of mating or injection, but has not been observed until between 24 and 48 hours after the injection of copper salts or picrotoxin.

It seemed likely that other metals, particularly those associated in the periodic table with copper, might also be found to possess activity in producing ovulation. I have therefore examined the action of some of these, and attempted further to elucidate the way in which such ovulation is produced.

MATERIALS AND METHODS

Adult virgin or parous does of heavy breeds were used. They were usually isolated for a month or more, and some had been used for ovulation tests not less than three weeks previously. They were kept in a warm room. Such rabbits respond regularly to gonadotrophic preparations.

Laparotomy for inspection of the ovaries was carried out under sterile conditions either 24 or 48 hours after injection. After an animal had been used for three such tests, it was killed, as sensitivity, at any rate to gonadotrophic extracts, tends to diminish with repeated use [Hill, Parkes & White, 1934]. Haemorrhagic and cystic follicles have generally been ignored, undoubted ovulation points being taken as the only criterion of response.

All injections were made into an ear vein; the substances were dissolved in distilled water and given in a volume of from 0.5 to 5 ml. The anti-gonadotrophic serum referred to later in the text was prepared by injecting female rabbits with 25 mg. per day of a pyridine extract of acetone-desiccated ox pituitary (AP24B), the serum being taken approximately 2 months after the beginning of injections. Sera of this type have predominantly an antiluteinizing activity, will inhibit ovulation in the mated doe and inhibit implantation and cause abortion if given during pregnancy [Parkes & Rowlands, 1936]. They also cause atrophy of the reproductive organs of the male rat, and inhibit ovulation in the pubertal female rat [Rowlands, 1937].

RESULTS

Copper salts.

The finding of the previous workers, that copper salts will cause ovulation in the oestrous rabbit, was first investigated. 15 mg. of copper acetate

were found to stimulate ovulation in 11 out of 15 rabbits, 2 rabbits dying soon after injection (Table I). Of those surviving, therefore, 11 out of 13, or about 85%, ovulated, showing between 1 and 11 ovulation points, with an average of 4.6 points per rabbit. The ovulation appeared in every way normal, and occurred within 24 hours of injection. In one experiment the actual time of ovulation was determined by keeping the two does under urethane anaesthesia and examining the ovaries at frequent intervals from the 11th hour after injection. The earliest ovulations observed occurred at 15 hours 50 minutes and 16 hours 40 minutes; 5 and 3 ruptured follicles respectively were present in the two cases at 17 hours 30 minutes after injection. There was thus some delay, in comparison with the post-coitum latent interval, but it was not so marked as that reported by previous workers.

Copper sulphate and copper alanine were also effective in causing ovulation. The dose at which these compounds produce a response is not far below the lethal dose (cf. Table I), and they do not appear to be as potent, in terms of copper content, as the acetate. An attempt to produce ovulation by feeding the acetate and alanine to two rabbits, in total doses of

Table I. *The effect of salts of copper and cadmium in causing ovulation in oestrous rabbits (intravenous injection)*

Substance	Dose mg.	No. of rabbits	No. ovulating	No. dead within 24 hours
Copper acetate	5	1	0	0
	10	1	1	0
	15	15	11	2
Copper sulphate (cryst.)	10	1	0	0
	30	2	1	1
	40	2	0	1
	60	1	0	1
Copper alanine	15	1	0	0
	20	1	0	0
	25	3	2	1
	35	1	1	0
	50	2	0	2
Cadmium acetate	5	1	0	0
	15	2	1*	1
	20	1	0	1
Cadmium chloride	5	1	0	0
	15	2	2	0
	20	1	1**	0

* this rabbit ovulated although pregnant

** " " " " pseudo-pregnant

approximately 100 mg. given during the course of a day, was unsuccessful. The compounds did not prove lethal in this dosage.

Cadmium salts.

Ovulation also took place after the administration of cadmium chloride and acetate. In one case in which ovulation occurred, and in one case in which it did not, the ovaries and Fallopian tubes were abnormal in appearance, and showed extension haemorrhages. Owing to the use of a few animals which had not been isolated for a sufficient period, two of the rabbits in which ovulation was caused by salts of cadmium were pregnant and pseudo-pregnant respectively, conditions in which it is normally more difficult to produce ovulation than during oestrus. As with copper salts, ovulation occurred within 24 hours of the injection.

Salts of other metals.

Various salts of barium, cobalt, gold, iron, manganese, nickel, silver and zinc were found to be ineffective in causing ovulation (Table II). The tests with these substances were not extensive, but as lethal doses of many of them were given it seems improbable that an effective dose-level has been missed. The failure with zinc confirms the findings of Fevold *et al.* [1936] and Saunders & Cole [1938]. It is clear, as Marshall *et al.* [1939] point out, that ovulation caused by non-specific substances is not the consequence of a general shock to the central nervous system such as might be supposed to occur with near-lethal doses of some of the heavy metals. Gold, for instance, caused death with convulsions in a dose of 20 mg. of the chloride, but did not cause ovulation in a lower dose.

Further experiments with copper acetate.

In an attempt to find out how copper salts cause ovulation, a series of rabbits were hypophysectomized as an acute experiment by partial decerebration, according to the method of Fee & Parkes [1929]. The operation, rapidly carried out under deep urethane and ether anaesthesia, was followed by an injection of copper acetate. Eight such animals were prepared, but none survived as long as 12 hours. The injection of copper acetate evidently reduced considerably the usual survival time of such preparations. It was thus not possible to determine by this method whether the presence of the animal's pituitary gland is necessary for the reaction. It did not seem likely that rabbits hypophysectomized by a more orthodox technique would prove any more resistant to the toxic effects of copper, since the operation is always severe and the injection must be made immediately afterwards, before atrophic changes begin in the ovary.

A second line of attack was made by the use of the antigonadotrophic serum referred to on p. 64. This serum, given in a dose of 10 ml., regularly inhibited ovulation after mating, but about 1 ml. was sufficient to inhibit

Table II. *Various metallic salts which did not cause ovulation in oestrous rabbits (intravenous injection). One rabbit was injected at each dose level*

Substance	Dose mg.	Period elapsing before laparotomy
Barium chloride (cryst.)	5	48 hours
	20	"
	40	Died within 24 hours
Cobalt acetate	10	24 hours
	20	"
Ferric chloride (anhydr.)	5	48 hours
	20	Died shortly after injection
Gold chloride	10	48 hours
	20	Died in convulsions after injection
	20	" " " "
Manganesec chloride	15	24 hours
	30	"
Nickel acetate (cryst.)	5	48 hours
	20	"
	40	"
Silver acetate	5	"
	10	"
	20	Died shortly after injection
Silver sulphate	5	48 hours
	20	"
Silver vitellinate	10	24 hours
	20	"
Zinc acetate	10	"
	15	"
	20	"
	25	"
	40	"

ovulation after the injection of gonadotrophic extract in a dose sufficient otherwise to cause 100% response. This difference in amount of antiserum required under the two conditions is concordant with Hill's observation [1934] that the rabbit pituitary apparently frees a much greater quantity of gonadotrophic material immediately after mating than is actually needed to cause ovulation.

The serum was injected intravenously, immediately after the injection

of 15 mg. of copper acetate. The results are shown in Table III. Ovulation was inhibited with as little as 0.25 ml. of serum, but occurred in one of the two rabbits receiving 0.1 ml. The control rabbits listed in Table III are those which were injected with copper acetate in previous experiments and which survived for 24 hours after injection. It is evident from these results that the ovulation produced by copper salts is not a result of direct action on the follicles, but must involve the animal's own gonadotrophic

Table III. *The inhibitory effect of an antigonadotrophic serum on the response of oestrous rabbits to 15 mg. of copper acetate. Both substances were given intravenously*

Amount of serum ml.	No. of rabbits	No. ovulating
—	13	11
5.0	4	0
2.5	2	0
1.0	2	0
0.5	2	0
0.25	2	0
0.1	2	1

secretions. Nevertheless, it is curious that the antiserum should have been effective in such small amounts.

Since the response may be inhibited by relatively small amounts of antiserum, the amount of gonadotrophin involved must be considerably less than that released by the pituitary gland after coitus. This might be adduced in favour of the idea that synergism occurs between the metallic salts and normally circulating hormones. The haemorrhagic state of the ovaries seen in a few rabbits suggests, further, that a process occurs which is not entirely normal. Nevertheless, I incline to the belief that the action of the metallic salts is to stimulate the pituitary gland to increased production of the ovulating substance. While the possibility that synergism occurs between the injected salts and normally circulating hormones has not been ruled out, the known ability of the pituitary gland to secrete gonadotrophin under a variety of stimuli provides a more solid basis for an explanation of the facts than the postulation of an otherwise undemonstrated synergism.

SUMMARY

Salts of copper and cadmium will cause ovulation in oestrous rabbits. This ovulation may be inhibited by the simultaneous injection of an antiserum produced in rabbits to ox pituitary extract, and is probably due to stimulation of the rabbit's anterior pituitary gland by the metallic salts.

Salts of barium, cobalt, gold, iron, manganese, nickel, silver and zinc did not produce ovulation.

I am indebted to Dr. I. W. Rowlands for the preparation of the anti-gonadotrophic serum, and to Dr. O. Rosenheim for the preparation of copper alanine.

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THE EXCRETION OF FREE OESTROGEN DURING UTERINE BLEEDING

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EXCEPT during phases of menstruation, when as a rule little or none is demonstrable, the urine of normal women of reproductive age almost always contains fat-soluble oestrogenic hormone, mostly in a state of combination [Palmer, 1937, 1939*b*]. The amount excreted varies, and as a rule is equivalent to from 4 to 30 $\mu\text{g.}$ of oestrone; amounts up to 200 $\mu\text{g.}$ may, however, occur during one or two days of the mid-intermenstruum. Oestrogen in free form is occasionally and transiently present, and it can be demonstrated only by the extraction and assay of both free and combined hormone in consecutive, complete, 24-hour urine specimens. As a rule it occurs during the menstrual cycle only at the time of ovulation and during menstrual bleeding—if it can be demonstrated at all.

While the amount of combined oestrogen gradually and steadily increases until term, only negligible quantities of free hormone are excreted during pregnancy. Its presence in the urine of gestation is probably due to unpreventable hydrolysis (bacterial or enzymic) of part of the large amount of combined hormone before the urine is extracted. On the other hand, free oestrogen increases considerably at the onset of either normal or premature labour [Cohen, Marrian & Watson, 1935; Marrian, 1936; Palmer, 1938]. This increase may be related to the uterine bleeding that is associated with parturition, and to the increase in free oestrogen which occurs at the time of normal menstrual bleeding. The fact that free oestrogen was also excreted during successive phases of bleeding, in a woman suffering from a granulosa cell tumour of the ovary [Palmer, 1939*a*], suggested the following clinical study to test whether its occurrence is a normal concomitant of uterine bleeding.

METHODS

Tests for free and combined urinary oestrogen were made on more than a hundred specimens, and in particular on successive specimens from two women suffering from menorrhagia and cystic hyperplasia of the endometrium. These two patients (Nos. 1 and 2, Table I) were closely

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studied. Hormone therapy was withheld during a preliminary period of observation, when basal metabolic rates were determined and endometrial tissue was obtained for histological examination. Endometrium was taken from many regions of the uterus, at intervals of not less than a week, by the use of a serrated tubular curette to which continuous

Table I. *The excretion of oestrogen in pathological and normal states*

Case	Free oestrogen*	Combined oestrogen*	Clinical condition
	$\mu\text{g.}$	$\mu\text{g.}$	
Case I. 'Hyperoestrinism'	4.8	8.0	24th day of bleeding
	8.0	120.0	31st day of bleeding
	4.8	24.0	38th day of bleeding
	0.0	8.0	Not bleeding
	0.0	12.0	Not bleeding
Case 2. 'Hyperoestrinism'	8.0	12.0	1st day of bleeding
	0.0	8.0	8th day of bleeding
	0.0	28.0	Not bleeding
	8.0	48.0	Not bleeding
	0.0	15.0	Not bleeding
	8.0	12.0	33rd day of bleeding
	8.0	12.0	18th day of bleeding
Granulosa cell tumour	86.6	—	Bleeding
Ovarian carcinoma	23.0	0.0	Bleeding
Fibroid uterus	12.6	0.0	Bleeding
Onset of normal menses	8.0	0.0	Last premenstrual day
Pregnancy terminated at 4½ months	14.6	8.0	Bleeding 1st day p.p.
	22.5	9.0	Bleeding 2nd day p.p.
Twin pregnancy terminating spontaneously at 3½ months	200.0	100.0	Bleeding 1st day p.p.
	100.0	20.0	Bleeding 2nd day p.p.
	220.0	0.0	Bleeding 3rd day p.p.
	29.0	0.0	Bleeding 4th day p.p.

* All values for oestrogenic hormone are calculated as $\mu\text{g.}$ oestrone-equivalent excreted per 24 hours.

suction was applied. Sections of the biopsy specimens were routinely stained with haematoxylin and eosin, and examined to determine whether or not ovulation had occurred and to estimate the extent to which the endometrium had responded to stimulation by the ovarian hormones. The patients were studied further by assaying 24-hour urine specimens for oestrogenic and gonadotrophic hormone. The collection of each specimen was completed at 8 a.m. of the day an endometrial specimen was obtained. The gonadotrophic hormone extraction was carried out according to a tungstic acid precipitation procedure described by Freed &

Hechter [1936]. The oestrone-equivalent of free and combined oestrogen was estimated according to the procedure outlined by Palmer [1939 b]. It should be stated that all extractions for free oestrogenic hormone were started within 8 hours of the completion of the 24-hour specimen.

CLINICAL DETAILS

The more important clinical details of the two closely studied patients are as follows:

Patient No. 1. Aet. 35. During the first 4 years of her menorrhagia this patient had been hospitalized 4 times for therapeutic curettage; an intra-uterine exposure to radium (amounting to 400 M.C.H.) was also made. Relief was only temporary after each of these measures. Other forms of treatment tried and found to be of temporary benefit only were intramuscular injections of the patient's own blood and the administration of chorionic gonadotrophic hormone. Desiccated thyroid and progesterone produced no definitely beneficial effects. The latter therapy was started by giving 5 mg. of progesterone every 2 days during an episode of bleeding. It was stopped after the 5th dose because the bleeding became more profuse. Endometrial curettage with the suction curette seemed effective in stopping the bleeding within two days, and no effect of the progesterone was observed in the specimen of endometrium obtained. For the next three months the patient was free from bleeding except for two 3-day periods at intervals of 30 and 52 days respectively. Then, beginning 21 days later, the patient experienced 35 days of continuous bleeding which, following a suction curettage, again stopped. A second series of progesterone injections was given while bleeding was absent (5 mg. daily until a total of 65 mg. had been given). A specimen of endometrium was obtained the day following the last injection of progesterone. Bleeding followed this procedure and again assumed haemorrhagic proportions. The biopsy specimen showed definite effects of the progesterone treatment. This patient had thus received as much, if not more, relief from her symptoms by endometrial curettage as by hormone therapy.

Patient No. 2. Aet. 17. This patient, now 18 years old, began menstruating when 13, missed a period one year later, and then bled continuously for 4 months. Since then she has had irregular prolonged cycles of bleeding, the interval between successive phases of bleeding averaging 12 days. Sixteen months before entering the clinic she was hospitalized for a therapeutic curettage. After the curettage she did not bleed for 40 days, the longest period of amenorrhoea she had experienced in $3\frac{1}{2}$ years. As a patient of the clinic she received, on successive occasions, desiccated thyroid, chorionic gonadotrophic hormone, and one course of 40 mg. of

progesterone administered during 2 weeks of a phase of bleeding. An attempt to obtain a specimen of endometrium following this period of progesterone therapy failed. Thyroid was probably responsible for an increase in the basal metabolic rate from -15 to -3 .

No form of hormone therapy used seems to have corrected the pathological condition of the patient, and endometrial curettage has been the most efficient measure for the relief of her symptoms.

RESULTS

All specimens of endometrium obtained from both these patients showed evidence of hyperoestrogenization. During a period of observation of patient No. 1, five 24-hour urine specimens were obtained, 3 during phases of bleeding and 2 in the absence of bleeding. Seven 24-hour specimens were obtained from patient No. 2, 4 during phases of bleeding and 3 in the absence of bleeding.

Moderate amounts of oestrogenic hormone were readily demonstrable in every specimen of urine from these two patients (Table I). Free oestrogen was readily demonstrable, but as a rule only in specimens collected during uterine bleeding. In their excretion of oestrogenic hormone these two patients therefore differed from normal women only in the fact that oestrogenic hormone was present in the urine in readily demonstrable amounts during phases of uterine bleeding.

Gonadotrophic hormone was not found in any of the specimens.

In other instances where 24-hour urine specimens were obtained from patients during phases of bleeding, the oestrogen present was either partly or entirely in its free form (Table I). A single 24-hour specimen from a 62-year-old woman suffering from an ovarian carcinoma, with uterine bleeding, was found to contain a moderate amount of free oestrogen. No combined oestrogen was present. On the other hand, a moderate amount of combined, but no free, oestrogen was found in 16 urine specimens collected from 5 women with secondary amenorrhoea. Free oestrogen was also not found in more than 100 urine specimens obtained from normally menstruating women during the intermenstrual interval.

CONCLUSION AND SUMMARY

Both normal and pathological uterine bleeding are thus associated with the presence of free oestrogenic hormone in the urine. For purposes of comparison, the uterine bleeding of abortion, labour and menstruation, as well as other types of uterine bleeding, may be considered to be similar physiological processes in so far as each is associated with increased uterine-muscle activity, endometrial shedding, and the excretion of free oestrogenic hormone. The relationship between uterine bleeding and the excretion of free oestrogen appears to have passed unrecognized hitherto.

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MECHANISM OF ACTION OF A PROGONADOTROPHIC SERUM

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THE capacity of antisera to certain gonadotrophic extracts to augment the response of the ovaries of the immature rat to these extracts is now well established. Progonadotrophic sera have been prepared by the administration of rat pituitary glands to rats [Katzman, Wade & Doisy, 1936, 1937] and by the continued injection of extracts of (a) sheep pituitary glands into sheep [Collip, 1937; Thompson, 1937; Rowlands, 1938,] (b) rat pituitary glands into rabbit [Sulman & Hochman, 1939], (c) ox pituitary glands into dog [Rowlands, 1939] and (d) pig pituitary glands into goat [Rowlands, 1938, 1939]. In addition, Simonnet & Michel [1939] have reported that antisera to extracts of horse pituitary gland and of the urine of ovariectomized women augment, in immature female mice, the gonadotrophic action of an extract of pig pituitary gland.

The mechanism of this augmentatory action is not yet understood. Thompson [1937] offered three possible explanations to account for this type of augmentation. Two of these, (a) that the serum contained follicle-stimulating properties, and (b) that the serum had the effect of delaying the rate of absorption of the extract thereby giving a more effective stimulation, he discarded on experimental grounds. The third explanation offered was that the augmentatory action of the serum might possibly be the result of the presence of an anti-substance to the pituitary antagonist [Evans, Korpi, Pencharz & Simpson, 1936].

In a recent paper Rowlands [1939] suggested that the progonadotrophic activity exhibited by the serum of a goat, which had been injected daily with an extract of pig pituitary gland, was due to its capacity to effect a partial but selective neutralization of the excessive amount of luteinizing hormone present in the same extract. It was argued that, in this way, there might be left a residue of gonadotrophic activity containing a more effective mixture of follicle-stimulating and luteinizing hormones for ovarian stimulation in the immature rat.

The results of further experiments, presented below, throw considerable doubt on the validity of all existing interpretations of the progonadotro-

¹ While working on a grant from the Medical Research Council.

phic effect, since they show that the augmentatory action of the serum tested could not be demonstrated in hypophysectomized test rats.

METHODS

Production of progonadotrophic serum. A goat (Goat 8) was injected subcutaneously once daily for 7 weeks (from 27.6.38–18.8.38) with 100 mg. of a gonadotrophic extract (AP74D) of pig anterior pituitary gland, prepared by aqueous alkaline extraction of the acetone-desiccated material. The yield of extract was 14.2%, so that the goat received daily the equivalent of about 700 mg. of the dried gland. The goat was bled under ether anaesthesia 48 hours after the last injection. 700 ml. of serum were obtained, which were Seitz filtered and stored at 2° C.

Preparation of dried globulin fraction. About 500 ml. of this serum were diluted with an equal volume of distilled water. A volume of saturated ammonium sulphate equal to that of the diluted serum was then added and the mixture allowed to stand at 4° C. for several hours. The precipitate (globulin fraction) was separated by filtration, dissolved in saline and re-precipitated in half-saturated ammonium sulphate. The re-precipitation was repeated a second time, after which the globulin was dissolved and dialysed against physiological saline at 4° C. When free of ammonium sulphate the solution was filtered and its concentration determined refractometrically; it was then stored in the frozen state. The final volume of the solution was approximately 220 ml., the globulin concentration being 4.61%.

The globulin solution was distributed into ampoules containing 5 ml. or 10 ml. and dried in high vacuum at -40° C. by the method of Greaves & Adair [1939]. The globulin fraction was stored in the dry state and for injection was dissolved in an amount of distilled water such that 1 ml. of the solution was equivalent to 2 ml. of the original serum.

The dried globulin fraction from 100 ml. of normal goat serum was prepared and used in the same way.

Biological assay. The serum and the globulin fraction were tested for their ability to increase the quantitative response of the ovaries of intact or hypophysectomized immature rats to injections of a standard amount (25 mg.) of the pig pituitary extract AP74D. The method of assay was similar to that previously described by Rowlands [1939]. As before, the extract and the serum were injected subcutaneously at different sites once daily for 5 days. The animals were killed 24 hours after the last injection and the ovaries and uterus were dissected and weighed after fixation in Bouin's fluid overnight. The ovaries were examined histologically; sections were cut serially, one in five being mounted and stained with haematoxylin and eosin.

RESULTS

Fractionation of progonadotrophic serum.

The results given in Table I show that the progonadotrophic activity of the serum of Goat 8 is present in the globulin fraction. This confirms the observations of Thompson [1937] and Katzman *et al.* [1939]. Thompson, who investigated the sera of a horse and a dog that had been injected with sheep pituitary extract, found that the activity occurred in the pseudo-globulin fraction, whereas the latter authors showed that the augmentatory factor in an iso-specific progonadotrophic serum from sheep fractionated with the eu-globulin.

Table I. *Fractionation of progonadotrophic serum (Goat 8). In all tests the serum and its fractions were injected simultaneously with a constant amount (25 mg.) of pig pituitary extract (AP74D). 1 ml. of globulin solution = 2 ml. of serum*

Serum fraction	Total amount injected ml.	No. of rats per group	Average weight of ovaries mg.
—	—	20	26
Whole serum	5	5	65
" "	5	5	52
Globulin "	2.5	5	67
Serum without globulin	5	5	35

The action of progonadotrophic serum on intact and hypophysectomized rats.

In these experiments groups of intact and hypophysectomized immature rats were injected with the standard dose of the extract of pig pituitary gland (AP74D) together with varying amounts (0.5 ml.-5 ml.) of the globulin fraction of the serum of Goat 8. The globulin fraction of normal goat serum was injected only into intact rats. The quantitative response obtained in the ovaries of these rats is shown in Table II and in Fig. 1.

It is seen that 2.5 ml. of the globulin fraction of the progonadotrophic serum increased the response of the ovaries from 26 mg. to 65 mg., that is by 39 mg., whereas 5 ml. increased the response by 62 mg. In hypophysectomized rats, however, 2.5 ml. of the globulin solution increased the response from 23 mg. only to 31 mg., that is by 8 mg., an increase not significantly greater than that produced in intact rats by the globulin solution from normal goat serum. The injection of larger amounts of the progonadotrophic globulin proved fatal to hypophysectomized rats, but it is evident that the maximum tolerated dose, which in intact rats increased the response of the ovaries by 39 mg., exhibited no significant progonadotrophic activity in the test animals deprived of their pituitary glands. The active globulin fraction itself, as shown by the test on hypo-

physectomized rats, was not directly gonadotrophic, and the negative result obtained from the similar test on intact rats indicated that it failed, by itself, to stimulate the secretion of endogenous gonadotrophin.

The injection of the progonadotrophic globulin fraction in amounts up to 2.5 ml. did not cause any qualitative alteration in the response of the ovaries of hypophysectomized rats injected with the extract of pig pituitary gland. The ovaries contained numerous medium-sized, heavily

Table II. *The effect of the globulin fraction of serum from Goat 8 on the response, given by the ovaries of intact or hypophysectomized rats, to 25 mg. of extract AP74D. The weight of ovaries of untreated intact rats is 10 mg.; that of untreated hypophysectomized rats 5 mg.*

Test animal	No. of rats injected	Total amount of globulin injected ml.	Average weight of ovaries mg.
Intact	20	—	26
"	5	0.5	37
"	5	1.25	57
"	5	2.5	65
"	5	5.0	88
"	5*	5.0	10
Intact	5	2.5†	28
"	5	5.0†	41
Hypophysectomized	6	0	23
"	5	0.5	20
"	7	1.25	25
"	6	2.5	31
"	3*	2.5	5

* Controls injected with globulin fraction only.

† Globulin from normal goat serum.

luteinized follicles resembling small corpora lutea, but, as far as could be determined from the histological examination of the ovaries, none had ovulated.

These observations leave the mechanism of the progonadotrophic action still quite unexplained in detail, but they seem to necessitate the conclusion that the augmentation produced is not concerned with the primary effects of the principles injected but with the output of hormones from the pituitary gland of the test animal, occurring as a secondary effect of the injections.

SUMMARY

1. The progonadotrophic activity appearing in the serum of a goat injected with an extract of pig pituitary gland was found to pass into the globulin fraction.

2. This globulin fraction, while augmenting the gonadotrophic activity of the same pig pituitary extract in intact rats, showed no progonaotrophic activity in hypophysectomized rats.

3. The role of the test animal's pituitary gland in progonaotrophic activity is being further investigated.

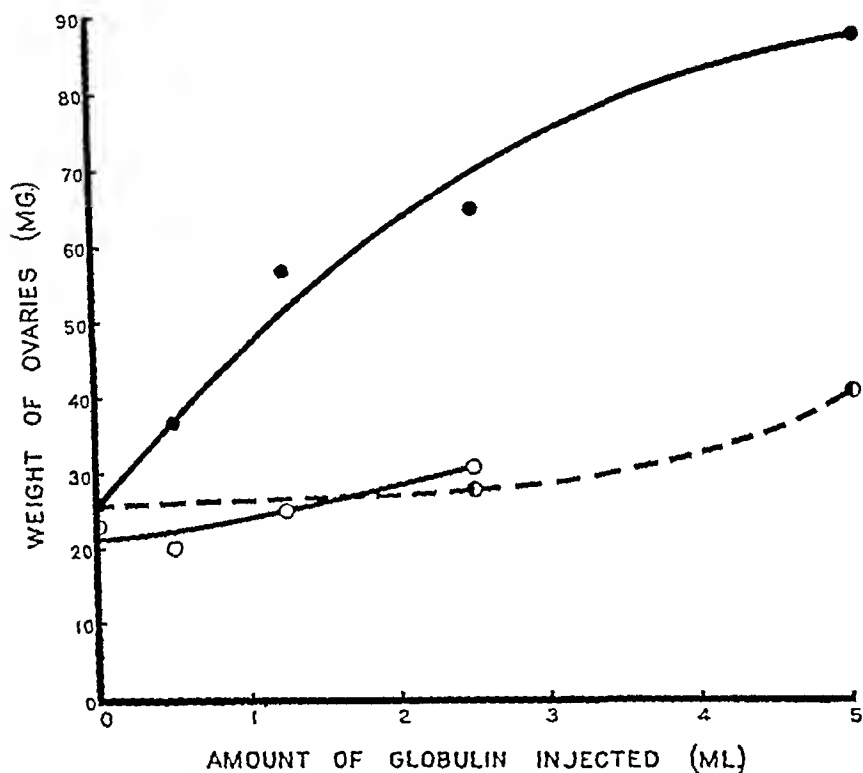


FIG. 1. The effect of varying amounts of the globulin fraction of the serum of Goat 8 on the ovaries of intact (●) and hypophysectomized (○) rats, and similarly of the globulin fraction of normal goat serum on the ovaries of intact rats (○). All rats were injected simultaneously with a constant amount (25 mg.) of extract of pig pituitary gland (AP74D).

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THE ISOLATION OF OESTRONE FROM OX ADRENALS

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IN 1930 Engelhart reported the preparation of lipoid extracts from adrenal glands, which produced oestrous and progestational effects in the uterus of the immature rabbit. These findings were confirmed by Callow & Parkes [1936], who showed that the active principles could be separated by the method of Allen & Meyer [1933]. In 1938 the isolation of progesterone, together with 3:20-*allo*-pregnanolone, was announced [Beall & Reichstein, 1938; Beall, 1938]. Oestrone has now been isolated from two ox adrenal concentrates. A preliminary report of this work appeared last year [Beall, 1939].

By the courtesy of N. V. Organon, Oss, Holland, two adrenal concentrates, both possessing a marked oestrogenic activity, were placed at the disposal of Dr. A. S. Parkes and made available to the author.

As a preliminary step, the first concentrate was saponified with hot alcoholic potash, the greater part of the oestrogenic activity appearing in the alkali-soluble fraction. Partition of this material between suitable solvents resulted in a further concentration of the active substances, which were then separated into ketonic and non-ketonic fractions. Following the sublimation of the ketonic material, *in vacuo*, and adsorption of the sublimate on an alumina column, a very active eluate was obtained. The residue from the eluate was treated with HgCl_2 and NH_4OH by the method of Beall & Edson [1936] for the isolation of oestrone from the urine of pregnant mares. Sublimation of the resulting gum followed by benzoylation gave oestrone benzoate which melted at $215-17^\circ$.

In the case of the second concentrate the saponified material was extracted with ether in a continuous liquid-liquid extractor. Practically all the oestrogens appeared in the ether-soluble, alkali-insoluble fraction. This was processed in a similar fashion to the alkali-soluble fraction of the previous concentrate and as a result oestrone was isolated as its 3:5-dinitrobenzoate which melted at $193-4^\circ$.

EXPERIMENTAL

Melting-points are uncorrected. Micro-analyses are by Dr. Weiler, Oxford.

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Preparation of the initial concentrate

The initial concentrate was prepared by N. V. Organon, Oss, from ox adrenals, by the method of Pfiffner, Vars & Taylor [1934]. The permutit-filtered alcoholic solution was evaporated to a small volume, and, after the addition of water, the alcohol was removed *in vacuo*. This gave an aqueous suspension and an oily residue. The latter was redissolved in alcohol, and reprecipitated with water, the whole process being repeated three times. Two such water-insoluble oils, OA7 (1,079 g.) and OA8 (1,280g.), each representing 3,000 kg. of ox adrenals, were used in the present investigation. OA7, assayed by the dilution method, had an oestrogenic activity equivalent to 110 mg. of oestrone; OA8, assayed by group assay, the equivalent of 120 mg. of oestrone.

Bioassays

All the bioassays reported in this paper were carried out by Dr. C. W. Emmens, National Institute for Medical Research, Hampstead, without whose generous co-operation it would have been impossible to have undertaken the investigation.

Owing to the large number of assays required, it was, in general, impractical to use the group response method. A system of decreasing dosage was therefore adopted, whereby the amount of material given to each succeeding mouse of a group of mice was halved, until the smallest dose giving a positive response was found. While the use of a single mouse for each dilution reduced the accuracy of the test, the method was found to be adequate for establishing the approximate activity of fractions and for following the partition of the active material in the various separations used. In expressing the assay results, the figures for the smallest dosage giving a positive response have been converted into an equivalent weight of oestrone, based on the fact that the majority of mice give a response with 0.1 μ g. of the hormone.

*OA7**Saponification*

357 g. of crude OA7 (representing 1,000 kg. of original tissue) were saponified in 1.5 l. of dry ether at room temperature by treatment for 16 hours with 300 ml. of 7.5% sodium methoxide in methyl alcohol. The product was separated in the usual way into the ether-soluble 'non-saps' and the alkali-soluble 'saps'. The latter were acidified and ether extracted, the ether being washed with 7% NaHCO_3 to remove a certain amount of acidic material. 176 g. of 'cold saps' were obtained in this way. The 'cold non-sap' fraction weighed 129 g. The oestrogenic activity was divided evenly between these two fractions, the potency of each

being equivalent to 18 mg. of oestrone. The bicarbonate washings, which were oestrogenically inactive, were rejected.

The 'cold saps' were dissolved in 1 litre of light petroleum and extracted ten times with 100 ml. of 90% methyl alcohol, the combined methyl alcohol washings being re-extracted once with 150 ml. of light petroleum. The residue (22 g.) obtained by taking the methyl alcohol solution to dryness under reduced pressure was dissolved in 500 ml. of 0.5 N KOH, which was saturated with carbon dioxide and ether extracted. The residue from the ether extract was an oil (16.7 g.) with a strong cresol-like odour. Repetition of the light petroleum-alcohol-bicarbonate process reduced this fraction to 6.7 g., designated 'phenols from cold saps'. It possessed all the oestrogenic activity of the total 'cold saps'.

Partition of the 'cold non-saps' between light petroleum and 90% methyl alcohol failed to concentrate the oestrogenic activity into the alcoholic phase. The fractions were recombined, taken to dryness and re-saponified by refluxing for 45 minutes with 700 ml. of alcohol and 100 g. of KOH dissolved in 100 ml. of water. The resultant 'non-saps' and 'saps' were separated and the 'phenols' of the latter were isolated as before and added to those obtained from the 'cold saps'. A residual oestrogenic activity, equivalent to 2 mg. of oestrone, remained in the 'non-saps'. On subjecting these to the light petroleum-methyl alcohol-bicarbonate process this activity passed into the resulting 'phenolic' fraction.

In view of the unsatisfactory results obtained by the cold saponification method, the remainder of the original concentrate (722 g.) was saponified by dissolving it in 2 litres of alcohol and refluxing for 1 hr. with 126 g. of KOH dissolved in 120 ml. of water. This procedure gave 499 g. of 'bicarbonate-washed saps' and 79 g. of 'non-saps'. The 'phenols' of the 'saps' were isolated as before. Those remaining in the 'non-saps' were obtained by dissolving this fraction in 1 litre of toluene, and extracting six times with 100 ml. of N NaOH, saturating the combined alkaline solutions with carbon dioxide and ether extracting. The ether extract, which was a light oil with a cresol-like odour, was added to the 'phenolic' fractions previously obtained.

Separation of the ketonic and non-ketonic 'phenols'

The combined 'phenols' were dissolved in 200 ml. of absolute alcohol containing 6 ml. of glacial acetic acid, and refluxed for one hour with 12 g. of Girard's reagent T. After being cooled, the mixture was poured into 800 g. of ice and water, containing 90% of the theoretical amount of sodium hydroxide required to neutralize the acetic acid, and extracted four times with ether. The combined ether extracts were re-extracted three times with 25 ml. portions of 35% alcohol, then washed with dilute

NaHCO_3 , then with water and taken to dryness. 15 g. of non-ketonic 'phenols' were obtained. The aqueous alcoholic phase was acidified by the addition of 15% by volume of concentrated HCl , and, after standing at room temperature for one hour, the mixture was extracted four times with ether. The ether was washed with dilute NaHCO_3 , then with water and taken to dryness. 1.62 g. of ketonic 'phenols', which had an oestrogenic activity equivalent to 25 mg. of oestrone, were obtained.

Isolation of oestrone from the ketonic 'phenols'

Partition of the ketonic 'phenols' between light petroleum and 90% methyl alcohol was ineffectual, as all the material stayed in the alcoholic phase. This was diluted to 70% alcohol and extracted four times with benzene. The combined benzene extracts were re-extracted five times with N NaOH . The soda washings were saturated with carbon dioxide and ether extracted to give 472 mg. of a gum which had an oestrogenic activity equivalent to 15 mg. of oestrone. While this was only 60% of the activity of the original ketonic 'phenols', the loss was offset by the degree of concentration obtained at this stage. Sublimation of this concentrate, *in vacuo*, at 100–170° C./0.05 mm. gave 184 mg. of gum, which on being subjected to a light petroleum-methyl alcohol-bicarbonate purification, gave 100 mg. of glass. No loss of oestrogenic activity took place during these stages.

The next step involved the use of an alumina column. In a preliminary experiment it was found that oestrone could be removed from a 10% benzene-light petroleum solution by running it through activated alumina. Elution with benzene-light petroleum mixtures, benzene or boiling benzene-alcohol failed, however, to recover any of the hormone. This was probably due to the presence of free alkali in the alumina. On the other hand, oestrone acetate which had been similarly adsorbed on alumina could be recovered by washing the column with 50% benzene-light petroleum. The 100 mg. of glass was therefore acetylated (acetic anhydride-pyridine on the water-bath) and the product was dissolved in 10 ml. of benzene. Addition of 90 ml. of light petroleum gave an opalescent solution which was run through a 5 g. alumina column. The column was then washed with 50% benzene-light petroleum, the amount of oestrogens appearing in the eluate being determined colorimetrically in terms of oestrone. The 10% benzene eluate was colorimetrically negative. The first 200 ml. of 50% benzene brought through the equivalent of 6.1 mg. of oestrone, the next 300 ml. the equivalent of 2.0 mg. and a further 100 ml. the equivalent of 0.4 mg. of oestrone. Washing with 85 ml. of pure benzene gave only a further trace of chromogenic material.

Sublimation of the residue (34 mg.) from the 50% benzene-light petro-

leum eluates, at 150–170° C./0.05 mm. gave 28.5 mg. of gum which were saponified and treated with 3:5-dinitrobenzoyl chloride in pyridine solution on the water-bath. An attempt to crystallize the product by dissolving it in 2 drops of benzene, adding 2 ml. of light petroleum and allowing it to stand overnight in the icebox was unsuccessful as this gave only a few crystals melting at 170–80°. The mother liquors were taken to dryness, dissolved in 25% benzene-light petroleum and run through a 2 g. alumina column. This was washed with a total of 450 ml. of 25% benzene-light petroleum and 200 ml. of 50% benzene-light petroleum but no oestrogens (determined colorimetrically) appeared in the eluate. The alumina was then extracted with a mixture of boiling benzene-acetone-alcohol containing two drops of glacial acetic acid. Washing was continued until the extracts were colorimetrically negative. No crystalline material could be obtained from this eluate so that it was saponified and, on subliming at 150–170° C./0.05 mm., gave a gum. This was treated with HgCl_2 in alkaline solution and then with concentrated NH_4OH [cf. Beall & Edson, 1936]. The resulting precipitate was hydrolysed with alcoholic HCl and the product was sublimed at 170° C./0.05 mm. Benzoylation of this sublimate gave crystals melting at 215–17° alone or when mixed with authentic oestrone benzoate. The details of the procedure used were as follows:

The 15 mg. of sublimate from the saponified dinitrobenzoate were dissolved in 10 ml. of N NaOH in a centrifuge tube and 1 ml. of 5% HgCl_2 was added. The mixture was heated for 30 min. in a boiling-water bath, cooled, and 5 ml. of concentrated NH_4OH were added. This gave a large gelatinous precipitate which was centrifuged down and washed twice with 10 ml. of 1% alcoholic acetic acid. The residue was then boiled for 5 minutes with 15% (by volume) alcoholic HCl , cooled, and the excess acid was neutralized with NH_4OH . After making the mixture just acid with acetic acid, it was taken to dryness under reduced pressure. The residue was taken up in water to dissolve the inorganic salts and filtered, the filter being washed thoroughly with boiling water and then extracted well with boiling alcohol. Traces of mercuric salts in the alcoholic washings were removed by saturating these with H_2S . The alcohol was removed, *in vacuo*, the residue was redissolved in alcohol and then filtered through kieselguhr. Sublimation of the alcoholic residue at 170° C./0.05 mm. gave 5.3 mg. of crystalline material which when recrystallized from aqueous alcohol melted at 242–7°. This was benzoylated with benzoyl chloride in pyridine solution. The product was dissolved in ether and washed with dilute HCl , NaHCO_3 and water. It was dried over KOH in a vacuum desiccator to remove traces of benzoyl chloride. The resultant gum was sublimed at 150–180° C./0.05 mm. and gave 1.3 mg. of crystals melting

at 215–17° alone or 216–17° when mixed with authentic oestrone benzoate (m.p. 215–16°). A group of ten mice, each receiving a single dose of 0.5 μ g. of the sublimate, gave an 80% response, whereas a control group receiving a similar dose of authentic oestrone benzoate gave a 90% response.

OA8

OA8 (1,280 g., containing the equivalent of 120 mg. of oestrone) was saponified by refluxing for one hour with 600 ml. of 20% alcoholic potash. The mixture was then diluted with 6 litres of water and extracted with ether for 18 hours in a continuous liquid-liquid extractor. The rate of flow of the solvent was approximately 2 litres per hour. The aqueous and ether phases, which were processed in a similar way to those obtained on saponifying OA7, yielded 430 g. of 'bicarbonate-washed saps' and 625 g. of 'non-saps' respectively. The latter fraction was much larger than that obtained from OA7 where the saponified material was separated in a funnel.

The 'saps' and 'non-saps' were partitioned by the light petroleum-methyl alcohol-bicarbonate process. The resultant 'phenolic' fractions were combined and the partition process was repeated. This gave 33 g. of a light oil, possessing a cresol-like odour, which was separated into ketonic and non-ketonic fractions with Girard's reagent T. The resulting 'phenolic' ketones weighed 2.38 g. and had an oestrogenic activity equivalent to only 5 mg. of oestrone.

In the meantime the light petroleum-soluble fraction of the 'non-saps' had been separated into ketones and non-ketones. The ketones were partitioned between light petroleum-70% alcohol and 70% alcohol-benzene in order to recover any *allo*-pregnanolone present [Beall, 1939]. The low oestrogenic potency of the 'phenolic' ketones suggested that some activity might have remained in this light petroleum fraction of the 'non-saps'. The benzene-soluble and alcohol-soluble residues were therefore taken up in toluene and extracted six times with N NaOH in order to recover any residual phenolic material. The alkaline extracts were combined, saturated with carbon dioxide and ether extracted. The ether residue, which weighed 292 mg., had an oestrogenic activity equivalent to 25 mg. of oestrone.

An attempt to sublime this material at 170° C./0.05 mm. was unsuccessful as it resinified. The resin was dissolved in dilute NaOH, which was saturated with carbon dioxide and ether extracted. The ether extract yielded 76 mg. of gum. This was acetylated, dissolved in 50% benzene-light petroleum and run through a 5 g. alumina column. The column was washed with a total of 800 ml. of 50% benzene-light petroleum and the

residue from this eluate was saponified. 41 mg. of gum were obtained. This was subjected to the $\text{HgCl}_2\text{--NH}_4\text{OH}$ reaction and gave 23 mg. of gummy material. Sublimation of this at $170^\circ\text{C./0.05 mm.}$ gave 11 mg. of crystals contaminated with a little gum, which were treated with 3:5-dinitrobenzoyl chloride in pyridine. Crystallization of the product from 3 drops of benzene and 2 ml. of boiling methyl alcohol gave 7 mg. of crystals melting at $193\text{--}4^\circ$ alone or when mixed with authentic oestrone 3:5-dinitrobenzoate (m.p. $193\text{--}4^\circ$). 2 mg. of the crystals were saponified in ether by sodium methoxide. Recrystallization of the product from aqueous alcohol gave oestrone which melted at $252\text{--}3^\circ$ alone, or $253\text{--}5^\circ$ when mixed with authentic oestrone (m.p. 255°). A group of mice receiving two injections of $0.05\text{ }\mu\text{g.}$ of the isolated oestrone gave a 50% response, whereas a control group receiving the same dosage of authentic oestrone gave an 80% response.

Isolated 3:5-dinitrobenzoate. Found C, 64.7%; H, 5.46%.

Oestrone 3:5-dinitrobenzoate. Requires C, 64.6%; H, 5.21.

SUMMARY

Oestrone has been isolated, in one case as its benzoate and in the other case as its 3:5-dinitrobenzoate, from two ox adrenal concentrates.

The author is greatly indebted to N. V. Organon, Oss, Holland, who, through Dr. A. S. Parkes, National Institute for Medical Research, London, made this investigation possible by supplying the initial concentrates. Grateful acknowledgement is also made to the Medical Research Council for a grant towards the cost of the work.

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17-KETOSTEROID, ANDROGEN AND OESTROGEN EXCRETION IN THE URINE OF CASES OF GONADAL OR ADRENAL CORTICAL DEFICIENCY

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THE discovery that material with the activity of the gonadal hormones can be extracted from human urine early led to the plausible and attractive hypothesis that the level of this urinary excretion might give an indication of the level of secretion by the gonads. An obvious line of critical attack on this idea was that of examining subjects whose gonads had been removed and in whom the complete absence of gonadal secretion should entail the absence of either androgenic or oestrogenic activity in the urine. A fair volume of work on this line has already been done, but with contradictory results. McCullagh, Cuyler & Frawley [1932], McCullagh, McCullagh & Hicken [1933], McCullagh & Reushaw [1934], Kochakian [1937] and Feinier & Rothman [1939] were unable to detect androgens in extracts from the urine of eunuchs. Bingel [1935] appears to have been the first to obtain positive results, using the sensitive biological test of comb-growth in the capon after local application of the extract, and his report was followed by results of the same kind recorded by Koch [1936, 1937], Kenyon, Gallagher, Peterson, Dorfman & Koch [1937], Chou & Wu [1937], Hansen [1937 *b*, 1938] and McCullagh [1939]. It is fair to say that the amount of androgen found has tended to rise with improvements in the technique of extraction: the highest value yet recorded is given by Hansen [1938] for one subject, viz. c. 2180 'percutaneous capon units' per litre, which, using his conversion factor, is 22 I.U./l. The presence of oestrogenic material in the urine of eunuchs has been reported by Bingel [1935], Eng [1936], Hansen [1937 *a*], Quental [1937] and Kenyon *et al.* [1937]. The values recorded by different workers are not comparable, but they vary from one-third to eight times the values for normal men obtained in the same laboratories.

Few examinations of the urinary excretion of androgens by ovariectomized women have been reported. Dingemanse, Borchardt & Laqueur [1937] found low values by biological assay in three cases, and more recently Hamblen, Ross, Cuyler, Baptist & Ashley [1939] used Oesting's colorimetric method to determine androgens in the urine of women after hysterectomy and ovariectomy and recorded that little change occurred

after operation. Investigations of the oestrogen excretion are more numerous: Laroche, Simonnet & Huet [1933] found oestrogens in varying amounts in the urine of about half the women they examined who had been castrated surgically or by X-rays, and estimations have also been recorded by Eng [1936] and Frank, Goldberger & Salmon [1936].

The present investigation, a preliminary account of which was given in a lecture by one of us [R. K. Callow, 1938], has extended to the examination of eleven eunuchs and eighteen ovariectomized women. Without exception, material with androgenic activity has been detected in the urine of gonadectomized subjects. Oestrogenic material has been detected in the urine of all cases, with the exception of a few ovariectomized women; this last statement must, however, be taken with the reservation that our methods of extraction of oestrogens from normal urine are not very satisfactory. As a whole, the results support Bingel [1935], who was the first to come to the conclusion, after examining selected human subjects, that a demonstration of sex hormones in the urine could not be regarded as evidence of the functional capacity of the gonads. In spite of this clear lack of confirmation of the primary hypothesis and the demonstration that some of the active material in urine was of extra-gonadal origin, it remained to be seen whether, when the androgen and oestrogen excretion of groups of cases was examined, quantitative differences could be found which were sufficiently large or constant to allow urines of gonadectomized or eunuchoidal subjects to be distinguished from normal urines.

For reasons which have been expounded elsewhere [Callow, Callow, Emmens & Stroud, 1939], our chief attention has been given to the colorimetric assay of 17-ketosteroids in urine extracts, as giving a measure of the degradation products of the male hormone series. The figures obtained for androgens and oestrogens by biological assay, although we attach less weight to them, and although the latter in particular are of uncertain significance, are comparable among themselves, having been obtained with uniform techniques of extraction and estimation.

As an extension of this work, we considered an alternative to the hypothesis of the gonadal origin of 17-ketosteroids or androgens in urine, namely, that they might be derived wholly from the adrenal cortex. In this event the urinary excretion of 17-ketosteroids in cases of Addison's disease would be very small. A condition of complete deficiency of both gonads and adrenal cortex, such that the occurrence in the urine of 17-ketosteroids from other sources could be investigated, cannot be studied in man, but the nearest approach to this condition was observed in a case of severe pituitary deficiency.

Detailed clinical data on many of the cases whose urines we examined

were made available to us by the generous co-operation of a number of physicians. Since, however, it proved impossible to draw any general conclusions from these data, they are not included in this paper.

METHODS AND NORMAL STANDARDS OF COMPARISON

The methods of extracting urine used in this investigation have been the routine methods of separate or combined hydrolysis and extraction previously described [Callow, Callow, Emmens & Strond, 1939]. The colorimetric assay of 17-ketosteroids was carried out by the method of Callow, Callow & Emmens [1938]. The methods of biological assay were those previously employed in this laboratory [Emmens, 1939; cf. Callow, Callow, Emmens & Strond, 1939]. All urines were adequately preserved. As far as possible, collections were made over a continuous period of three days, and the results of assay expressed in terms of the daily output, but in a number of cases this could not be done, and the results, marked with an asterisk in the tables, are expressed in terms of content per litre.

The figures for normal subjects, which we take as a basis for comparison, are those for a series of hospital patients and for mixed collections of urine [Callow, Callow, Emmens & Strond, 1939]. The values obtained for 17-ketosteroid excretion were 3.5–15.0 mg./day (mean 9.05 mg./day) for men, and 1.7–12.6 mg./day (mean 6.75 mg./day) for women. Comparable figures were obtained by Chou & Wang [1939] who found 4.3–21.0 mg./day, (mean 10.7 mg./day) for normal men and a lower range of 0.35–10.5 mg./day for hospital patients, the values in the latter being lower in proportion to the state of malnutrition and duration of illness. Only a few values of androgen excretion by normal subjects have been determined in this laboratory, the values ranging from 6.5 to 110 International Units per day for men and from 2 to 50 I.U./day for women. Dingemans *et al.* [1937] give the figures 15–170 I.U./l. for men and 30–60 I.U./l. for women. Since the average volume of urine excreted daily is about one litre, figures expressed in terms of I.U./l. are comparable with those in terms of daily excretion. Koch [1939] gives the figures 40–100 I.U./day for young men and 30–100 I.U./day for young women. Kochakian [1937] reported the excretion of 6–36 'capon units' per 24 hours by young men, a figure which we [Callow, Callow & Emmens, 1939] unjustifiably assumed to be too low. Subsequent assessment of these values by comparison with androsterone, however, gives the figures 14–102 I.U./day (personal communication from Dr. Charles D. Kochakian). The principal workers in this field are, therefore, in substantial agreement as to the magnitude of the androgen excretion and its wide variations in young normal subjects. Figures for old people given by the same authors cover a lower range, and androgens may even be undetectable in urine from such subjects.

It is more difficult to say what are the values for excretion of oestrogens by normal subjects. Our own figures for bulk collections indicate figures of 10-30 I.U./l. for men's urine and 50-160 I.U./l. for women's urine, using the same method of biological assay in every detail [cf. Emmens, 1939] as has been employed in this investigation. Differences in methods, particularly of biological assay, rule out any comparison with figures from other laboratories: our own figures are only comparable among themselves. It may, however, be mentioned that the relatively greater excretion of oestrogens by women is consistent with results elsewhere, e.g. Koch [1939] gives the average values 270 I.U./day for women and 100 I.U./day for men.

RESULTS

Eunuchs and eunuchoids

The figures for eunuchs are given in Table I. Of nine values for 17-ketosteroid excretion, six are derived from normal colour reactions, and all these are within the normal range, but all except one are below the average figure for normal men. The values of androgen excretion are likewise within the normal range, but all tend to be low and are below the average for normal men. The values of oestrogen excretion are low and probably all below the normal average. We have not been able to discern any relation between the levels of 17-ketosteroid, androgen or oestrogen excretion and age or the time since castration in this series.

Table I. *17-Ketosteroid, androgen and oestrogen excretion in the urine of eunuchs*

Case no.	Subject	Age	Time since castration	17-Ketosteroid mg./day	Androgen I.U./day or *I.U./l.	Oestrogen I.U./day or *I.U./l.
1	†E.C.	19	5 yr.	10.9	18	12.5
	"	20	6 yr.	9.4	—	8.5
2	F.D.	—	—	8.2	20	c.4
3	†D.S.	38	19 yr.	8.4	21	8
	† "	—	—	7.2	15	6
4	T.C.	—	—	7.7	14	3.5
5	W.H.	—	—	6.7	12.5	7
6	G.B.	45	21 yr.	Free: (2.7) Combined: 6.4	Trace	c.4
				5.0	11	3
7	F.F.	45	20 yr.	(8.3)	7	6
8	A.R.	46	22 yr.	(3.1)	11	2
9	†F.C.	45	10 yr.	—	10	1.5
10	L.	24	1 yr.	—	c. 25*	c.6*
11	A.J.K.	42	—	Free: — Combined: —	not detectable	2
					17	6

() Nominal figures for colour reactions not typical of 17-ketosteroids.

† Data previously published [Callow, Callow & Emmens, 1939].

It would be expected that in eunuchoids, when no signs of testicular activity are clinically discernible, the 17-ketosteroid and androgen excretion would resemble those of eunuchs. The figures in Table II show that exceptions may occur. Case 12 is in marked contrast to the others and well above the normal average. Case 13, which did not show symptoms of complete deficiency, may be excluded. Cases 14, 15 and 16, in spite of discordant capon assays on different specimens of urine of case 14, give figures more or less in agreement with those of eunuchs. Kenyon *et al.* [1937], Carmichael & Kenyon [1938] and Dorfman & Hamilton [1939] found low values for the androgen excretion of eunuchoids. Eunuchoidism is a condition the aetiology of which is obscure, and evidently assay of urinary androgens may give information and assist in differentiating certain types.

Table II. *17-Ketosteroid and androgen excretion in the urine of eunuchoids*

Case no.	Subject	Remarks	17-Ketosteroid mg./day	Androgen I.U./day
12	A.S.	age 39 (1)	11.5	31
	"	—	16.0	c. 100
13	H.B.	age 44	13.1	80
14	S.A.	age 47	—	83
	"	—	5.6	6
15	F.F.	age 56	9.4	8
16	B.W.	—	(3.8)	5

() Nominal figures for colour reactions not typical of 17-ketosteroids.

Ovariectomized women

The figures obtained for ovariectomized women are given in Table III. Unfortunately there was uncertainty about the period of collection of the urine samples in many cases, but probably no serious error is involved in assuming that the values per day are similar to the values per litre. On this basis it appears that most of these subjects gave figures for 17-ketosteroid excretion within normal limits, and three were actually above the average. The androgen excretions are generally below normal. On the whole, however, the range of values is strikingly similar to that for eunuchs, although the values for normal women are generally lower than those for normal men.

Case 17, in which the 17-ketosteroid and androgen values are of the order obtained in cases of hirsutism associated with adrenal hyperplasia, is anomalous, but no item in the clinical history could be clearly related to these high values.

The values for oestrogen excretion were mostly very low, but in Cases 26 and 31 they were not far below the normal range, and Case 19 gave a normal value, for which the clinical history suggested no explanation. In

the two cases in which examination before and after operation was possible, clear falls in the values for androgen and oestrogen excretion were observed. Attempts to estimate 17-ketosteroid excretion after operation yielded values artificially raised owing to interference by material giving with the reagents a brown colour not typical of 17-ketosteroids, and it is probable that the 17-ketosteroid excretion fell in Case 34 as well as in Case 33. This observation is not in agreement with those of Hamblen *et al.* [1939].

Table III. *17-Ketosteroid, androgen and oestrogen excretion in the urine of ovariectomized women*

Case no.	Subject	Age	Time since operation	17-Ketosteroid mg./day or *mg./l.	Androgen I.U./day or *I.U./l.	Oestrogen I.U./day or *I.U./l.
17	B.L.	23	4 yr.	19.4*	135*	7*
"	"	23½	4½ yr.	21.4	63	5
18	Ha.	32	4 m.	—	17	0.25
"	"	33	16 m.	10.0*	59*	5*
"	"	"	18 m.	7.4	6	c.4
19	Co.	22	3 m.	6.8*	2.3*	100*
20	Hu.	38	?	6.6	16	5
21	E.M.	27	6 wk.	6.5*	17*	5*
22	F.L.	19	9 m.	6.2*	24*	4*
23	L.	53	4 yr.	5.5*	12*	4*
24	†F.Y.	32	2 yr.	5.0*	2.5*	7*
25	E.B.	38	2 m.	(9.0)	3	4.5
26	G.S.	53	3 wk.	(5.4)*	1*	19*
27	V.C.	43	1 yr.	—	14	7
28	Ca.	34	1 m.	—	13	4
29	X.	(young)	2 wk.	—	6	4
30	L.T.	54	1½ yr.	—	4.5	4
31	B.R.	?	2 wk.	(2.8)*	1.7*	30*
32	F.R.	56	27 yr.	—	c.1	3.5
"	"	"	"	—	2	2

Cases examined before and after operation

33	M.C.	45	Before	11.1	21.5	31
"	"	"	2 wk. after	(5.9)	10	7
34	E.H.	38	Before	8.6	36	19
"	"	"	2 wk. after	(10.6)	5	3

() Nominal figures for colour reactions not typical of 17-ketosteroids.

† Radium sterilization; all other cases were surgical.

Cases of Addison's disease

The figures for cases of Addison's disease are given in Table IV. The most marked feature is the preponderance of 'nominal' figures for 17-ketosteroid excretion, i.e. the frequent presence of substances which masked the colour reaction with *m*-dinitrobenzene. It is doubtful whether any significance is to be attached to this circumstance, but, as in such cases the figure obtained is an upper limit to the true value, it is clear that

the excretion of 17-ketosteroids tends to be below the average for normal subjects, and the same is true of the androgen and oestrogen excretions. It has to be assumed that these subjects are suffering from an insufficiency rather than a complete lack of endogenous adrenal cortical secretions, which is compensated for to a greater or lesser degree by administered extracts, for we cannot be certain, as we can in the case of gonadectomized subjects, that the glands are absent or that they fail to function.

Table IV. *17-Ketosteroid, androgen and oestrogen excretion in the urine of cases of Addison's disease*

Case no.	Subject	Sex	17-Ketosteroid mg./day	Androgen I.U./day	Oestrogen I.U./day
35	J.P.	M.	(7.5)	7	c. 35
36	C.B.	M.	(3.6)	18	11
37	J.M.	M.	(3.5)	23	c. 2
38	C.	F.	7.5	26	21
39	N.G.	F.	5.3	9	47
	"	"	(9.8)	39	137
40	V.	F.	(4.6)	13	11.5
41	J.F.	F.	(3.1)	1.5	8
	"	"	(2.3)	20	8

() Nominal figures for colour reactions not typical of 17-ketosteroids.

Case of pituitary deficiency

The figures in Table V are those obtained for the 17-ketosteroid and androgen excretion in a case of pituitary deficiency caused by pressure on the gland by a tumour, a case under the charge of Mr. W. R. Henderson at the National Hospital, Queen Square. It can be assumed that in the

Table V

Case no.	Subject	Remarks	17-Ketosteroid mg./day	Androgen I.U./day
42	J. M. (male)	Boforo operation	(2.6)	0.8
		About one week after operation	(6.2)	4
		About five weeks after operation	(7.1)	15

absence of stimulation from the pituitary, neither the adrenal cortex nor the testes were providing the precursors of the urinary 17-ketosteroids and androgens. Consistently with this, in the colorimetric assay, the colour given lacked the selective absorption characteristic of 17-ketosteroids, and the androgenic activity of the urine extract was only just detectable. A slight rise in the figures occurred 1 week after removal of the tumour, and, a month later, restoration of pituitary function brought with it a rise of the figures practically to normal levels, although substances interfering

with the colorimetric assay were still present, but in relatively small amount. Naturally, in a case of this kind, other endocrine organs besides the gonads and the adrenal glands are affected, and absence of 17-ketosteroids and androgens provides no certain indication of their normal source.

DISCUSSION

The comparison of these groups of subjects with normal men and women in respect to the excretion of substances allied to the steroid hormones gives a somewhat equivocal result. Separately, neither gonadal nor adrenal cortical deficiency results in the disappearance of 17-ketosteroids, of androgens or of oestrogens from the urine, except for the absence of detectable oestrogenic activity in the urine of some ovariectomized women. On the other hand, in both classes of subject the excretion of these materials is generally lower than is normal, but the range of values overlaps the range of values for normal subjects.

If it is assumed that the 17-ketosteroid and androgen excretion of patients with Addison's disease is derived partly from administered adrenal cortical extract, then the values we found indicate that without this administration the excretion would be very low. It seems likely that both the secretions of the gonads and of the adrenal cortex contribute to the urinary 17-ketosteroids, but that the adrenal cortex is the larger contributor. In the case of oestrogens it would appear that both glands may contribute, but that possibly the gonads, and especially the ovaries, are more important sources.

The results obtained do not provide clear evidence in favour of the supposition that deficiency in either the gonads or the adrenal cortex is balanced by increased activity of the other.

Evidence of the reciprocal relations of the adrenal cortex and the testes in laboratory animals has been provided by a number of investigations in the last few years (cf. Deanesly [1928], Cramer & Horning [1937], Davidson [1937], Hall & Korenchevsky [1938], Burrill & Greene [1939], Gersh & Grollman [1939]), but the conclusion which might be drawn from these observations, that loss of the testes may be partly compensated for by increased androgenic activity of the adrenal cortex in rats and mice, is not necessarily applicable to the human species, which is in one respect peculiar, in that it excretes relatively large amounts of androgenic 17-ketosteroids in the urine. Hamblen *et al.* [1939] have recently, on the basis of evidence from colorimetric assays of 17-ketosteroids in urine of patients with amenorrhoea, or past the climacteric, concluded that an increased production of androgens by the adrenal cortex compensates for regressive alterations in the sexual systems of ageing women, or, in other words, 'that the adrenal cortex becomes the gonads of the aged'. They

also consider that their data suggest that androgenic titres rise as a late effect of ovariectomy. Our data from Cases 33 and 34 certainly show a fall of 17-ketosteroid and androgen excretion immediately after ovariectomy, and our data from other patients after ovariectomy do not support the suggestion that a great increase in the production of androgens by the adrenal cortex is by any means a general phenomenon; only one such patient in our series, Case 17, gave high figures both for 17-ketosteroid and androgen excretion. It can only be concluded that a factor at present unrecognized may come into play in some of these cases, but not in others, and lead to a high degree of compensatory activity of the adrenal.

After having isolated three 17-ketosteroid compounds from the urines of normal men and women, and having identified two of these, viz. androsterone and aetiocholan-3(α)-ol-17-one, as excretory transformation products of testosterone [N. H. Callow, 1939; Callow & Callow, 1939; Callow, Callow & Emmens, 1939], we thought it possible that the remaining compound, *transdehydroandrosterone*, might be identified as the characteristic excretory product from the adrenal cortex. Hirschmann [1939] reported that these three compounds could be obtained from the urine of ovariectomized women in a yield only slightly less than that obtained by ourselves from normal women's urine. We [Callow & Callow, 1940] have examined the urine of a eunuch (Case 1) and have again found the same three compounds, but with an increased proportion of *transdehydroandrosterone*. Thus, even more specific chemical examination does not clearly distinguish between the contributions of the testis and the adrenal cortex to the 17-ketosteroids excreted in the urine. The increased proportion of *transdehydroandrosterone* is consistent with the hypothesis of compensatory activity of the adrenal cortex; as, however, the comparison was made between the urine of a eunuch showing a level of total 17-ketosteroid excretion higher than others and bulk collections of urine from normal men, and we have no knowledge of the range of variation in composition in either class, these observations cannot be regarded as positive evidence in support of this idea.

The practical object of this work was to investigate the possibility of using assays of androgen and oestrogen in the urine as a laboratory aid to diagnosis. The results, taken in conjunction with those reported in a previous paper [Callow, Callow, Emmens & Stroud, 1939], which demonstrated the wide range of variation in the excretion of 17-ketosteroids or androgens in patients not suffering from adrenal or sexual disorders, and the uncertainties of oestrogen estimation, show that the method has, as yet, no diagnostic value, except in certain special cases. Oestrogen assays have some value when they are made on a series of samples by uniform techniques of extraction and assay, but the method is too laborious and

expensive for common use, except where approximate determinations of large amounts are concerned. The estimation of 17-ketosteroids can be carried out with fair accuracy, and can replace biological estimation of androgens, but clear diagnostic significance can only be given to the very high figures which occur in cases of adrenal cortical tumour. In combination with other determinations and with clinical evidence it seems possible that assays of 17-ketosteroids can give some assistance in the diagnosis of pituitary insufficiency and of Addison's disease, in which low values would be confirmatory of other evidence, whilst average or high values would, in the absence of other strong evidence, be inconsistent with these diagnoses.

SUMMARY

1. Extracts of the urine of eunuchs, eunuchoids, ovariectomized women, sufferers from Addison's disease, and a case of pituitary deficiency have been examined for 17-ketosteroids, androgens and oestrogens.
2. 17-Ketosteroids and androgens were found without exception in the urine of 11 eunuchs and 18 ovariectomized women. The average amounts are below those for normal men or women, but individual figures come within the range of variation found in normal subjects.
3. Oestrogens were detected in the urine of all the eunuchs and most of the ovariectomized women. With one exception, an ovariectomized woman, the level of excretion was below normal.
4. The level of excretion of 17-ketosteroids and androgens is generally low in subjects with Addison's disease.
5. 17-Ketosteroids and androgens were low in a patient with severe pituitary deficiency, but the excretion rose practically to normal values when the patient recovered after operation.
6. It is concluded that urinary 17-ketosteroids and androgens may be derived from both the gonads and the adrenal cortex. The results are consistent with the hypothesis that absence of the gonads leads to compensatory activity of the adrenal cortex in some cases, but do not yield positive evidence in its favour.
7. The independent diagnostic value of determinations of urinary 17-ketosteroid excretion is limited to cases of adrenal cortical tumour; in conjunction with other evidence they have confirmatory value in cases in which lowered adrenal cortical function is suspected.

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THE RATE OF LOSS OF ACTIVITY OF ANTI-GONADOTROPHIC SERUM *IN VIVO*

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ROWLANDS in unpublished data has shown that antithyrotrophic activity is almost completely absent from the serum of a rabbit, sensitized by prolonged injection with an extract of ox pituitary, nine weeks after the injections were discontinued.

In a recent paper Rowlands & Spence [1939] have demonstrated the rate of appearance and subsequent loss of antihormone activity in human patients treated with an extract of pregnant mare serum, in which the loss of antigonadotrophic activity is nearly complete three months after the period of treatment. This result parallels in general, though not in detail, that obtained with antithyrotrophic serum in the rabbit, and seems to indicate that a period of months elapses before antihormone activity is completely absent in actively immunized animals.

Passive sensitization of animals of a different species from that of the donor, on the other hand, as will be seen below, is lost much faster.

METHODS

Antiserum. The globulin fraction used [Rowlands & Harington, 1937], which was active against the gonadotrophic principle of pregnant women's urine, was obtained from a goat (goat 1) immunized by means of injections of an extract ('Pregnyl') from this source.

Assay. Immature female rats weighing 40–50 g. were injected subcutaneously once daily for five days with a standard amount of 0.2 mg. of an extract of the urine of pregnant women (U.P.10). Twenty-four hours after the last injection the ovary and uteri were dissected, and after fixation overnight in Bouin's fluid they were weighed.

The administration of such an amount of U.P.10 by this method has been shown previously [Deanesly, 1935] to produce ovaries weighing 35–40 mg. The weight of the ovaries of untreated rats of this body-weight is 10–12 mg.

Other groups of immature rats, immunized by a single subcutaneous injection of antigonadotrophic serum, were subsequently, after different intervals of time, tested for their ability to inhibit the effect of 1 mg. of U.P.10.

The requisite amount of antiserum to neutralize 1 mg. of U.P.10 has previously been shown by Rowlands & Parkes [1937] to be 0.0125 ml.

The globulin fraction from this amount of serum was made up to one ml.

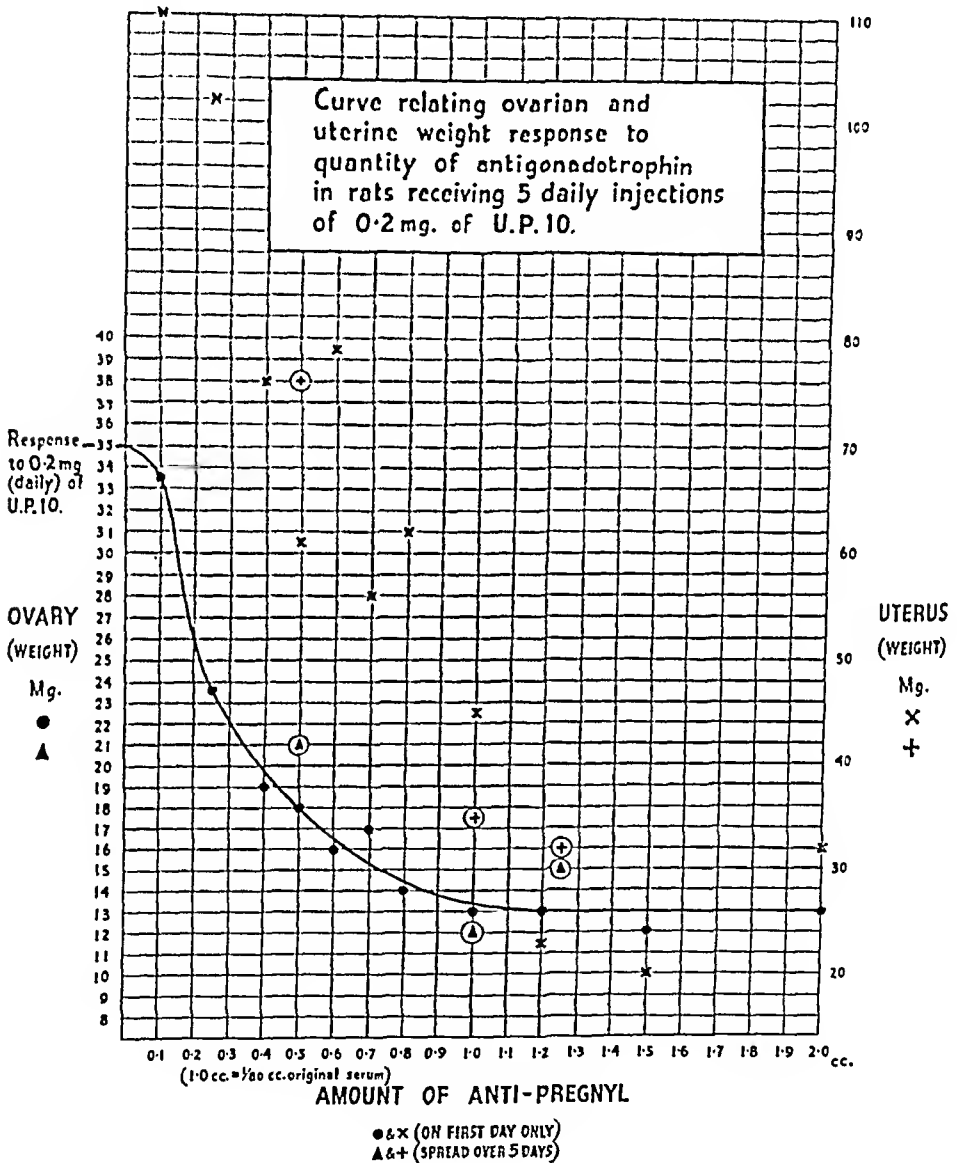


FIG. 1.

with distilled water, and administered subcutaneously to seven groups of ten immature female rats weighing 35–45 g. Subsequently, after different periods, five consecutive daily injections of 0.2 mg. of the urinary extract (U.P.10) were begun, and the effect assayed by the increase in the weight of the ovary. (Table I and Fig. 1).

RESULTS

Comparison of action of antiserum in single and divided doses.

In Fig. 1 and Table I is shown the effect of different amounts of the antigonadotrophic serum administered in five equal doses given simultaneously with five injections of the extract of urine of pregnant women U.P.10. It is seen that a total of 0.0125 ml. of the antiserum completely inhibits the effect on the immature rat's ovary produced by 1 mg. U.P.10.

Table I. *Ovary- and uterus-weights obtained by the simultaneous administration of varying quantities of antigonadotrophic globulin with an optimum dose of urine of pregnancy extract*

Globulin antigonadotrophin dose	Weight of ovaries	Uterus weight
ml.*	mg.	mg.
nil	35.0	—
0.10	33.5	110
0.25	23.6	102
0.40	19.0	76
0.50	18.0 (21.0)†	61 (76)
0.60	16.0	79
0.70	17.0	56
0.80	14.0	62
1.00	13.0 (12.0)	45 (35)
1.20	13.0	23
1.25	— (15.0)	— (32)
1.50	12.0	20
2.00	13.0	32

* 1 ml. = 0.0125 ml. of original serum.

† Figures in parentheses obtained by administering the antigonadotrophin simultaneously with the U.P.10 in 5 doses, otherwise the antiserum was given in one dose with the first U.P.10 injection.

Moreover, it is shown that the efficiency of the antiserum is the same when it is injected as a single dose simultaneously with the first of the five injections of U.P.10. It seemed desirable, therefore, to determine the rate of elimination of the antiserum activity from the body of the rat by injecting the urine of pregnancy extract beginning at different intervals after the single injection of antiserum.

Rate of elimination of antigonadotrophic activity by the rat

As shown in Table II, the antiserum was injected to nine groups of rats, and subsequently, at varying intervals, each group was injected with 1 mg. of U.P.10 in the usual way. This interval is defined as beginning when the antiserum was administered and ending on the first day of injection of the urinary extract. The effect on the response elicited in the ovary is shown in Table II. From Fig. 1 it is possible to calculate the percentage of antiserum remaining active after any specified lapse of

time. The results given in Table II and Fig. 2 show that 50% of the activity of the antiserum is lost on the first day and subsequently a gradual loss ensues, leaving only 14% of its activity on the 10th day.

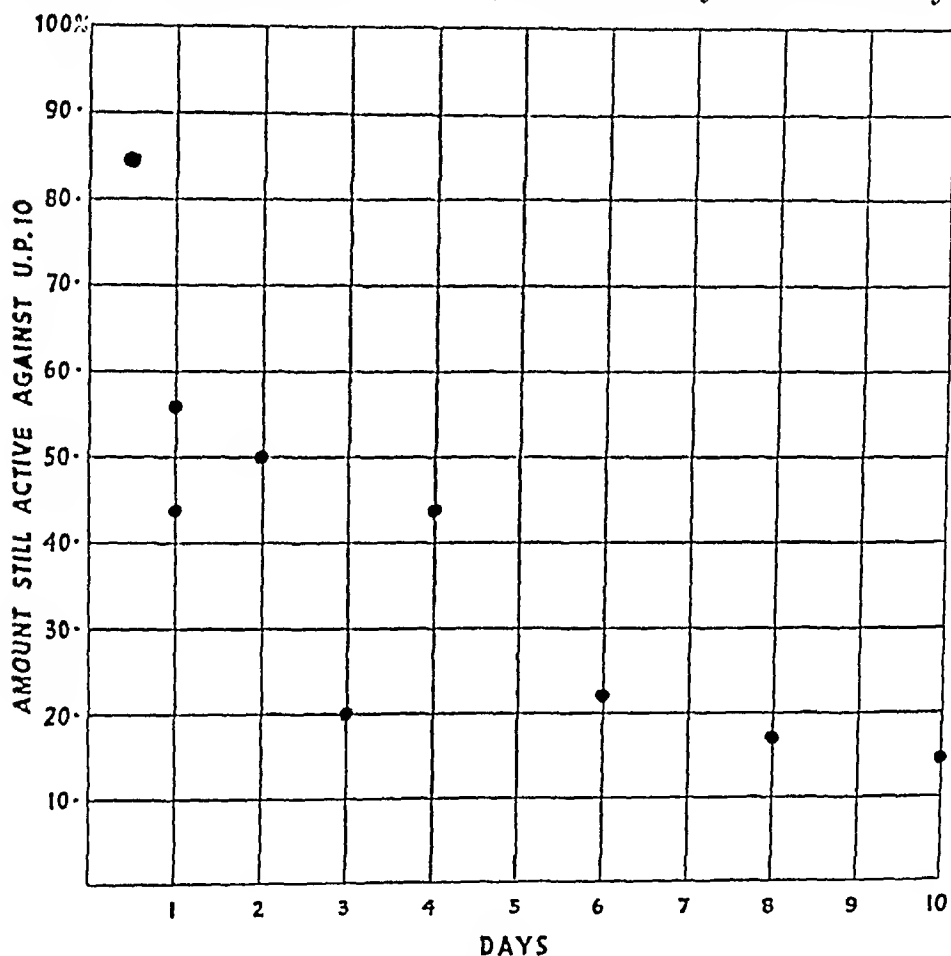


FIG. 2. Curve showing percentage of antigenadotrophic activity remaining after different time intervals.

Table II. *Table relating ovary-weight and percentage of antigenadotrophin remaining active after varying time intervals*

Days*	Weight of ovaries mg.	% Antigenadotrophic activity
0	13	100
$\frac{1}{2}$	14	84 $\frac{1}{2}$
1	18	51
2	18	50
4	19	44
6	25	22
8	28	17
10	30	14 $\frac{1}{2}$
—	35	0

* Period between injection of antigenadotrophic serum and first U.P.10 injection.

DISCUSSION

No direct comparison can be made between these results and the rate of loss of true passive immunity, as information is lacking on the disappearance of goat-serum in rats. It is, however, clear (from unpublished data) that the rate of loss of activity over the whole period of the experiment is of the same order as that for the disappearance of heterologous proteins in rabbits inoculated with staphylococcus antitoxin, and for the rate of loss of passive immunity in rabbits treated with horse antiserum reported by Glenny & Hopkins [1923, 1924].

In general, passively immunized animals lose the passaged antibodies sooner than those which are produced in their own system as a reaction to foreign antigens.

Great variability exists in this respect between different vaccines, but in general these results, when taken in conjunction with those of Rowlands & Parkes [1937], tend to support the view that antihormone formation is of the same kind as normal antibody formation.

SUMMARY

The rate of disappearance of the antigonadotrophic activity of goat serum from a goat immunized by an extract of the urine of pregnant women was investigated in rats.

It is shown that 50% of the activity of the antigonadotrophic serum is lost on the first day and that subsequently a gradual loss ensues, leaving 14% on the 10th day after injection of the serum into the rat.

My thanks are due to Dr. A. S. Parkes and Dr. I. W. Rowlands for assistance in the work.

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THE EXCRETION OF OESTROGEN AND PREGNANEDIOL BY PREGNANT AND PARTURIENT WOMEN

NORMAL AND TOXAEMIC CASES

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In previous studies [Hain, 1938, 1939] the author examined the range of the excretion of oestrogen in the urine of 47 pregnant women during the last four months of gestation. The data comprised the oestrogen excreted in untreated urine, and the combined forms of oestrone and of oestriol, the general inference being that a rise in the combined forms occurred between the fifth and sixth months of pregnancy, followed by a slight drop and a further rise during the last month. It was pointed out, however, that important facts might be obscured by considering only averages and that each case should be examined individually since the range of hormone output was so great, both low and high levels of excretion being unassociated with any abnormal symptoms in the outcome of gestation.

In the series referred to, the estimations of hormone concentration were made at monthly intervals and were, therefore, incapable of throwing any light on changes associated with the onset of labour. The present study has as its main object the investigation of these changes, and consequently frequent estimations were necessary during the last few weeks or days of gestation of both the oestrogenic and luteal hormones.

There can be no question that a fuller knowledge and appreciation of the various factors involved in normal function is urgently needed in order that the underlying causes of pathological function may be correctly assessed. Since not only in the non-pregnant but still more in the pregnant woman is there the interplay of not one but several hormones, an investigation which relates the timing and the amount of their secretion to a critical stage in the life of the patient is likely to throw light on their interaction and on their combined function, and to afford a standard (or standards, since individual variations in the amounts secreted are so great) with which pathological states can be compared. Patients in whom the balance between progesterone metabolism, as determined by pregnanediol glucuronide excretion, and oestrogen excretion has been such that birth has followed a normal course are placed alongside others in whom pregnancy has terminated disastrously.

The present series comprises 15 women in 10 of whom estimations were made every 3 (or at most 4) days during the last 2 or 3 weeks of pregnancy, and 5 others in whom they were made every day. In addition, one woman was followed throughout the whole of pregnancy at monthly intervals until the last month when weekly and, finally, daily analyses were made. Of the total of 16, 6 provide particulars of combined oestrone and oestriol only; in the remaining 10 cases the excretion of pregnanediol also was estimated and its relation to the combined oestrogens in the same specimen of urine was determined. In the majority, parturition either followed a normal course or the complications were minor ones; there were, however, three cases of still-birth (cases 62, 66 and 67), two of eclampsia or pre-eclampsia (58 and 67), one of pyelitis with disseminated sclerosis (68), one of mastitis (60), two with delayed second stage and commencing secondary uterine inertia (51, 62), and one in whom there was some delay in involution of the uterus associated with *B. coli* (61).

Hormone analyses were made either up to 1-3 hours before labour or during part or the whole of labour in 13 instances, and up to or within a few hours of parturition in 7 (cases 48, 57, 60, 64, 65, 67 and 68). The course of the decline in hormone output after parturition and during the commencement of lactation was examined in 9 cases (48, 60, 61, 62, 63, 64, 65, 66 and 67).

METHODS

Extraction of oestrogens from urine. The method of hydrolysis and separation of the oestrogens used in the previous series and described by Hain [1939] was again employed. This is based on the method of Cohen & Marrian [1934] but with modifications introduced by Callow [1936] in which continuous benzene extraction was substituted for ether extraction. By this method over 90% of oestrone (international standard) and approximately 60% of oestriol was recovered from a test aqueous solution. No allowance has been (or can as yet be) made for the augmenting effect of biologically inactive substances excreted in urine [Emmens, 1939], but since the same methods were followed throughout, the data afford valuable information for comparative purposes. In the present series the following minor alterations were made giving uniformity: (a) preparatory to the extraction of combined oestrone the urine was hydrolysed under reflux condenser for 1 hour only, and in the case of oestriol for 16 hours; (b) for both hydrolyses the urine was acidified in the proportion of 35 ml. concentrated HCl (commercial) per litre. A single 24-hour specimen of urine was used in all cases, and owing to the valuable co-operation of the matron and sisters of the (local) Homes where the patients stayed it was possible to be quite sure that the entire day's output was received. Of a

day's output, which generally consisted of 1500-1700 ml. urine, 250-500 ml. were set aside for the pregnanediol determination and the rest used for the estimation of the two oestrogens. Unless otherwise stated the post-partum urine included the first urine passed after parturition. All urines were worked up immediately on collection and no preservative was necessary.

As in the former series, groups of 20 ovariectomized mice were used for each test of oestrogenic activity, and whenever fewer than 30%¹ or more than 70%¹ showed full cornification in the vaginal smear, further groups of the same number were used until the reaction occurred within this range; frequently 40-80 mice were used on a single test. If fewer than 35% were cornified such a group was 'primed' again before use. It was sought in these ways to minimize the possible effect of a variation in reaction to previous injection and so to secure more accurate results. All newly ovariectomized mice were primed with strong oestrogen a fortnight before use.

Only full cornification was taken as a positive result and the findings were expressed in terms of milligrammes of the international standard as previously assayed in oil [Hain, 1938]. The main points of this assay and of that of oestriol, which was based on a specimen of crystalline oestriol kindly supplied by Professor Marrian, are shown in Table I.

Table I. *Assay of oestrone (I.S.) and crystalline oestriol in oil solution (4 injections in 36 hours)*

% mice positive	Oestrone (I.S.) mg.	Oestriol mg.
70	0.000108	—
60	0.000096	0.00100
50	0.000082	0.00085
40	0.000067	0.00070
30	0.000054	0.00050

In the majority of cases the oestrogenic activity of the untreated urine was also determined. The figures are given only in the first group as it was apparent that its concentration played little part in those changes which were associated with the onset of labour. It has been shown [Cohen, Marrian & Odell, 1936, and confirmed by the author] that oestriol glucuronide, which forms the major portion of the oestrogen excreted in pregnancy [Cohen & Marrian, 1934], possesses considerable oestrogenic activity in its combined form; it is thus mainly responsible for the oestrogenic effect obtained in the untreated urine of advanced pregnancy.

The androgenic activity of the urine was determined on capons, as pre-

¹ For oestriol these figures were 35 and 60% respectively.

viously, and a comparison made between the yield towards the end of gestation and two or three days after delivery. This will be reported separately.

Pregnanediol. The gravimetric method for the determination of sodium pregnanediol glucuronidate in urine as described by Venning [1937, 1938] has been employed in every instance. In order that quantitative values might be assured, a second precipitation with acetone and water was always made, as recommended by that author. The material recovered has been expressed in terms of pregnanediol by using the formula prescribed, and, as in the case of oestrogens, the output is estimated per 24 hours.

Charts. The same scale of values for each of the hormones investigated has been used on all the charts so that cases can be readily compared.

Clinical details of all patients are given in the tables.

RESULTS

Oestrogens only

In the six cases for which data of combined oestrone and oestriol alone are available (Table II, Figs. 1-6)¹ the following points are demonstrated:

(a) A marked rise in the excretion of combined oestrone occurred uniformly about one week before parturition: in three cases at 8 days before labour, and in two others at 6 and 9 days. In cases 51 and 57 (Figs. 1 and 5) some irregularity is observable in that the former did not exhibit a marked rise at 8 days and the rise in the latter was preceded by a similar peak at 15 days. These features may have been associated with other hormonal irregularities and be related to abnormalities in the patients' condition, as secondary uterine inertia necessitating low H.F. forceps delivery occurred in case 51 whose oestrone level was low and in whom no marked rise in output was observed during the 15 days covered; and in case 57 symptoms of mild pre-eclamptic toxæmia (B.P. 150 with some oedema of the ankles) were present from days 16-12. Abrupt changes in the level of combined oestrone excretion are not altogether rare (see case 48 below) but are not often accompanied by fluctuations of equal magnitude in the oestriol excretion. In this patient and in No. 58, in whom the condition of pre-eclamptic toxæmia necessitated hospitalization (see Table II), a high oestriol excretion occurred when the oestrone fell and vice versa.

(b) The second feature of this group is the drop in combined oestrone which followed on the rise and preceded the onset of labour by 3, 4, or 5 days. In 4 out of the 6 patients forming this series, labour commenced

¹ The tables, with two exceptions (Tables I and V), and all the figures are collected at the end of the article (pp. 126-140).

within 1-3 hours after collection of the last specimen of urine and in only two of these was there a further lowering in the output after the fourth day. The initial drop alone did not provide sufficient stimulus to bring about labour.

(c) The ascendancy of combined oestriol over combined oestrone is marked in all cases. Generally it shares in the rise which occurs in combined oestrone a few days before the onset of labour, but there does not appear to be a marked or consistent lowering of output prior to labour. This feature is held in common with combined oestrone. It seems obvious that other hormones and probably other factors play a more important part in the labour process than the level of combined oestrone and combined oestriol. This will be discussed later.

(d) With the exception of the two cases of high blood pressure (Nos. 57 and 58) the general outline of the combined oestriol curve follows, in the main, that for combined oestrone, but there is clearly no strictly quantitative relationship in the same specimen of urine. This is not surprising since it has been suggested that the conversion of oestrone into oestriol occurs under the action of progesterone [Pineus & Zahl, 1937] and such a relationship would necessitate a strict correlation between the three hormones. The later series in which the pregnanediol output has been estimated shows that this does not exist.

(e) In the women investigated the maximum daily excretion of combined oestrone was 1.9 mg. and of combined oestriol 24.9 mg.

(f) The amount of urine excreted daily has been shown in Table II, column 6, as it was thought that the peak of oestrogen excretion might coincide with a high urinary output. Only in cases 53 and 55 is it possible to see such a correlation in this series, and this may have been fortuitous.

(g) It was felt that more frequent analyses were necessary, and in subsequent cases these were generally performed daily towards the approach of labour.

Oestrogens and pregnanediol

This group consists of ten women of whom five may be considered to have presented no associated abnormalities, and five in whom serious symptoms either already existed or developed in the course of the investigation.

Case 48. As data are available for the whole course of pregnancy in patient 48, her case presents a valuable record for purposes of comparison. This was her fourth pregnancy, and the other children were healthy. Having been a scientific worker in this Institute before marriage she could be relied upon for conscientious co-operation. The first analysis of oestrogens was performed when her last menstrual period was 3 weeks overdue;

an Ascheim-Zondek test on this specimen of urine yielded a strong positive. Pregnanediol estimations were not commenced until the 5th month. The particulars in Table III and Fig. 7 show that during the first 4 months, although the combined oestrone was stationary the combined oestriol doubled itself monthly, from the 4th to the 8th months there was approximately ten times as much combined oestriol as combined oestrone excreted [confirming Cohen & Marrian, 1934]. It is not certain, however, that this relative uniformity actually existed as analyses at such long intervals gave no information as to daily variations.

During the last month of pregnancy (see Fig. 7a) the frequent estimations made (at 3-day intervals and then daily during the last fortnight) reveal a rhythmic rise and fall which, starting with a relatively slow swing covering 8 days, becomes more rapid, lasting 4 days and then 2 days. The rise and fall in combined oestriol are similar in character to those in combined oestrone but do not strictly coincide; a similar oscillation occurs also in pregnanediol, but the curve does not follow strictly either of the oestrogens. The general relationship of all three, however, is marked. It is of interest that the maximum pregnanediol excretion which was attained 13 days antepartum and again 4 days later was also reached at the 39th day antepartum (day 243). When reckoned per litre of urine the peak in pregnanediol excretion (60 mg.) occurred 8 days before labour at a time when the output per litre of oestrone was 1.6 mg. It is obvious that on the 274th day when the 24-hour excretion was 3.0 mg. oestrone and 60 mg. pregnanediol the kidneys played an important part in the process of hormone elimination, as on this day the values per litre of urine were only 1.5 and 29.5 mg. respectively.

It must be apparent to anyone examining the chart for any sure criterion that labour was imminent that no such criterion existed. Labour might have commenced on any day from the 8th antepartum onwards, as a comparison with the other charts will demonstrate. It is true that the urine passed during labour, though relatively rich in combined oestrone, contained little pregnanediol, but the same cannot be said in other cases, notably case 65 (*infra*). According to Cohen, Marrian & Watson [1935] labour is accompanied by a fall in the combined oestrone and oestriol and a rise in the free forms, but the connexion between the fall in combined oestrone observed by these authors and the appearance of free oestrone in the urine is not clear. Data are being accumulated which, it is hoped, will show the relation between the excretion of pregnanediol, combined oestrone, combined oestriol and free oestrone at the onset of labour.

A last noteworthy point in No. 48 is the high output of combined oestrone during labour. Labour lasted 17 hours and the urinary excretion (900 ml.) has been calculated on the basis of 24 hours. The postpartum

specimen, also, had a high oestrone content, but almost 3 litres of urine were passed.

Case 61 (Table IV, Fig. 8) shows much less irregularity than case 48 because the urine was examined at less frequent intervals. If allowance be made for the exaggerated scale for oestrone, there is, in this patient, a very fair degree of coincidence in the shape of the curves for the three hormones. The excretion of both combined oestrone and pregnanediol reached a very high level, the maxima being 5.3 mg. oestrone and 127 mg. pregnanediol. These, as also the maximum for oestriol (24.5 mg.), were reached 5 days before parturition. In the three days that followed, a sensational drop occurred in oestrone accompanied by a less marked fall in the level of pregnanediol, a combination of circumstances which suggests the approach of labour, but which has a parallel in case 48 on days 9-8 when a drop of 1.7 mg. oestrone occurred in a single day but without initiating the changes which herald parturition. As observed in case 48, the maximum excretion of oestrone and pregnanediol (5.3 mg. and 127 mg.) coincided with a high urinary output, viz. 2,580 ml.; per litre of urine the amounts were, respectively, only 2.0 and 49 mg. Thus, again, the kidneys played an important part in hormone elimination. It is interesting to note that 2 days before parturition witnessed the highest yield per litre of urine of both oestriol and pregnanediol: 17.0 mg. and 72 mg. respectively; thus, in spite of the lowered output of combined oestrone, the hormone excretion afforded no reliable indication of the imminent approach of labour. A similar peak though of much smaller magnitude occurred also per litre in case 48 two days before parturition, and in case 65 (*infra*) during the 18 hours which preceded parturition. Owing to the large amount of urine passed during the 24 hours following parturition (3,495 ml.) the hormone excretion is high, viz. 0.52 mg. oestrone, 8.0 mg. oestriol and 38 mg. pregnanediol. By 4 days after the birth there was no trace of pregnanediol, and only slight traces of oestrone and oestriol, the former being present in amounts normally found in the non-pregnant woman, viz. 5 μ g. per 24 hours. The delay in involution of the uterus noted in this case cannot be ascribed to any hormone abnormality and was no doubt attributable to *B. coli*; the condition cleared up within a week.

Case 65 (Table IV, Fig. 9). Daily analyses of hormone excretion were made in this patient during the last 7 days of pregnancy. Experience has shown that it is probable that the maximum hormone excretion occurred a little before estimations were started, but the data available permit one to place the case in the category of high excretors. Two outstanding features are (a) the very high excretion of pregnanediol 6 days before parturition (130 mg.), and (b) the extraordinarily high value for combined oestrone (and pregnanediol) during the 18 hours which preceded parturition, viz. 3.0

and 60 mg. These values are partly due to the high urinary output, as on day 6 the excretion of pregnanediol per litre was only 64 mg. and during labour the oestrone per litre of urine was 1.64 mg. However, on days 3 and 2 before parturition, when the amount of urine passed was considerably greater, the hormone values were only 50% of those stated above and indicate that on day 6 and at the onset of labour the *secretion* also was on a high plane.

The fall in the values for pregnanediol after day 5 suggested the near approach of parturition but subsequent yields, particularly those during labour, made it impossible to determine when parturition would occur.

On the whole, the curves for the three hormones show remarkable coincidence in outline. During the 24 hours immediately following parturition the fall in the level of oestrone is much more marked than that of pregnanediol, which has, however, completely disappeared from the urine before the 4th day postpartum, when there are still 13 μ g. of oestrone excreted. This is rather more oestrone than has been found in others on the 4th day postpartum, and, taken in conjunction with the high excretion during labour, may have had some connexion with the engorgement of the breasts observed on the 4th-5th days of the puerperium.

The remaining two cases in this group, Nos. 63 and 64, can be taken together as the data cover so short a period (Table IV). The output of both oestrone and pregnanediol in case 63 was high; 4.2 mg. during the day preceding parturition is an exceptionally high figure for oestrone (i.e. 3.3 mg. per litre of urine). Unfortunately, as only 328 ml. urine were passed during the 9 hours preceding labour no estimation could be made of its oestrone content; the pregnanediol recovered from 310 ml. gave the high yield of 123 mg. per 24 hours and indicates that a peak in excretion occurred at the onset of labour such as has been noticed also in case 65.¹ Similarly, the figure for oestriol shows a slight increase on that of the previous 24 hours, an increase which was certainly greater than that shown, as owing to lack of urine, the 310 ml. used for pregnanediol was used for oestriol estimation after having been treated with butyl alcohol which would remove most of the oestriol.

The rise in pregnanediol excretion immediately prior to parturition observed in case 63 (as also in case 65) again demonstrates how little indication of the approach of labour is provided by the analyses of these hormones in this form.

No specimen was obtained immediately postpartum, but it is interesting to note that as much as 60 μ g. of oestrone was being excreted 2 days after parturition.

The hormone output of case 64 (Fig. 10) is low but, as the analyses were

¹ This occurs also in case 62 (*infra*).

not commenced until 4 days before parturition, it is probable that the peak of excretion was already passed. It is noticeable also that on days 3 and 2 the urinary output was small; on day 3, on the basis of the yield per litre, the figure for pregnanediol reaches a more normal level (62 mg.). Furthermore, during the 24 hours immediately after parturition as much oestrone was excreted as in the same period preceding parturition, and fully twice as much oestriol, a fact which rather suggests faulty elimination, especially when taken in conjunction with the low urinary excretion. Four days after delivery the oestrone content had fallen to 5 μ g.

Examining this group of cases as a whole, one sees that the range of normality within even so small a group is very considerable, as the peak values given in Table V show.

Table V

	Oestrone	Pregnanediol	Oestriol
	mg.	mg.	mg.
Case 48	3	62	20
„ 61	5	127	24
„ 65	3	130	17
„ 63	4	123	9
„ 64	1	42	10

Allowance has to be made for the probability that higher values were reached by cases 63 and 64 as already stated.

Of the five patients showing abnormal features (Table VI), three gave birth to stillborn children, and in one instance (Case 66, Fig. 11) the child had been dead for about 3 weeks before the patient was admitted to hospital for artificial induction. The author is indebted to Dr. Susanne Paterson for particulars of the case and for permission to make the hormone analyses. During the 4 days prior to induction, the excretion of combined oestrone remained stationary at the low figure of 50–60 μ g. per diem, and the combined oestriol at 0.6 to 0.8 mg.; a 50% drop in the latter occurred on the day before parturition associated with a very low urinary output. Pregnanediol excretion was at the low level of 20 mg. 4 days before induction and fell still lower. On the day that labour was induced, the injection of 40,000 I.U. 'Progynon' may have been responsible for the 100% increase in the excretion of combined oestrone and pregnanediol per litre. The low recovery of injected oestrone on this day and that following is in keeping with the experience of many others [Siebke, 1930; Siebke & Schuschania, 1930; Zondek, 1931; Robson, MacGregor, Illingworth & Steere, 1934] but is very striking in this case when so large an amount of oestrone was administered.

In view of the presence of histidine in the urine of women giving a positive Ascheim-Zondek reaction [Kapeller-Adler & Haas, 1935], tests

for this substance were kindly carried out by Dr. Kapeller-Adler on three specimens from this patient, all of which gave a weak positive. This is of special interest owing to the length of time that the foetus had been dead.

Cases 62 and 67 (Table VI, Figs. 12 and 13) have in common not only the fact of still-birth but also an extraordinary rise in the excretion of pregnanediol during the last 8 or 10 days of gestation which culminated in a sudden outburst shortly before labour: in case 62 this occurred during the 12 hours immediately preceding labour and coincided with a large urinary output (2,440 ml. per 24 hours), thus again illustrating the part played by the kidneys in hormone elimination. Although the excretion for the 24 hours exceeded 100 mg. pregnanediol, actually per litre the figure was not high, viz. 43 mg., but during the fortnight from the 25th to the 10th day antepartum the excretion of pregnanediol had been much lower and was relatively constant in the neighbourhood of only 20 mg. per litre, or 40 to 50 mg. per diem. That the latter figure should suddenly touch 100 mg. suggests that there had been retention of progesterone due possibly to a faulty conjugation of the glucuronide in the liver, as suggested by Browne, Henry & Venning [1938]. Although no oedema or toxic symptoms were manifested by case 62 (the patient complained only of tiredness), the author feared that gestation was not following a normal course in view of the low level of pregnanediol and notified the physician to this effect three weeks before parturition. Reference to case 64 (above) shows that even lower values (30–40 mg. pregnanediol daily) may be encountered in the last few days of an entirely normal pregnancy, but data covering so short a period do not show from what levels (at, say, 12–8 days) excretion had fallen. A comparison with the figures 25–10 days before the end of gestation in cases 48 and 61 (the only ones in which data are available for this period) confirm the opinion that the values were below normal. Reference to the chart of eight normal cases given by Venning [1938] supplies further confirmation of this, although, in a personal statement, Dr. Venning affirms that, in her experience, entirely normal pregnancies have been associated with 'low values', but how low she does not say.

The figures for combined oestrone and combined oestriol do not show any marked abnormality except perhaps in the flatness of the falling curve from the eighth day onwards. Smith & Smith [1938] have found an association between low values of oestrone and toxæmia of pregnancy, but those of case 62 do not strike one as very different from, for example, case 55. In view of the fact that no foetal life was felt from 4 days antepartum, one examines (1) the hormone output immediately preceding the death of the foetus and (2) any associated deficiency occurring over a longer period which might account for the undersized condition of the

child at birth (wt. 5 lb. 6 oz.). Obviously the fall of oestrone synchronizing with a marked rise in the excretion of pregnanediol between days 9 and 4 antepartum was associated with the death of the foetus; and a low pregnanediol excretion, accompanied probably by a low secretion of progesterone over a prolonged period may have been largely responsible for its underdevelopment. It is of interest that it was after the child was dead that the excretion of pregnanediol reached its maximum, as though some inhibition had been released. That the placenta was functioning up to the time of labour is shown by the fact that a Hogben test kindly performed by the Pregnancy Diagnosis Laboratory on the urine passed at the commencement of labour symptoms gave a strong positive reaction.

The increased elimination of pregnanediol observed during the last 10 days of gestation in the case of No. 62 was encountered in case No. 67 but at a much greater level, and was associated with symptoms of severe toxæmia (Table VI, Fig. 13). On admission to hospital on 10 May her albumin was ++++; some improvement occurred under treatment but it was again +++ at labour (25 May), becoming negative the following day. The albuminuria was accompanied by high blood-pressure (172/96) which did not fall until 3 days before labour. Although no foetal heart was heard during the last fortnight of gestation, death may not have taken place until a short time before delivery, the patient's exceeding stoutness rendering it impossible to discern the heart beats. The weight of the child (10 lb. 8 oz.) shows full-term development.

The high hormone output—markedly excessive in the case of pregnanediol—is the outstanding feature of case 67. The fact that this does not apply to oestriol suggests that, as was probably the case in patient No. 62, only a small part of the progesterone represented by the colossal pregnanediol output was actually functioning (in the conversion of oestrone to oestriol) and that the major portion represents stored or faultily combined sodium pregnanediol glucuronide which was associated with the toxic condition present, either as a contributory cause or its effect. The highest value for pregnanediol given by Venning [1938] in a chart of eight normal cases was 100 mg./24 hours. At 11 days before parturition, when only 1,250 ml. urine were passed, case 67's output was 158.5 mg./24 hours, and 3 days antepartum 274 mg. The actual amounts of sodium pregnanediol glucuronide for the 2 days in question were 239 and 413 mg. respectively. When the pregnanediol is charted per litre of urine instead of per 24 hours, the curve is similar in outline to that given per diem. In this instance, therefore, the kidneys did not play so obvious a part in hormone elimination as in certain cases; nothing is known, however, of the urinary output prior to the 13th day, and if this was low, a different conclusion would be justified and a case made out for intoxication with

retained progesterone. A further noteworthy point is that even 24 hours before delivery the pregnanediol excretion was still very high: 134.5 mg., or 128 mg. per litre of urine; yet labour lasted only 2 hours. It was most unfortunate that, owing to an accident, the labour specimen was lost. The high excretion of combined oestrone during labour is an important feature—actually it is the same amount as was excreted by case 65 during labour.

In contrast with the weak positive reaction for histidine obtained by Dr. Kapeller-Adler in case 66 where the foetus had been dead for some weeks, the urine of this patient 13 and 11 days antepartum was entirely negative. Dr. Kapeller-Adler is reporting on a series of such toxæmias in which similar findings were obtained. The relation between gonadotrophic hormone and the liver demonstrated by Kapeller-Adler & Boxer [1937] suggests that abnormal liver function may exist in such toxæmias.

It would seem that the prevailing opinion is that late pregnancy toxæmias (eclampsia and pre-eclampsia) are associated with *low* levels of pregnanediol and therefore an insufficiency of progesterone. Browne *et al.* [1938] report one severe case in which the excretion was only 7.3 mg. per 24 hours at 250 days, and Weil [1938] states that in all cases of severe toxæmias, clinically pre-eclamptic, none of the compound (pregnanediol) could be recovered. Stover & Pratt [1939] also report low values. Those who have treated the condition with progesterone are Robson & Paterson [1937]—12 cases, and Marsden [1937]—8 cases; the former state that the maternal death-rate was lower than in a year when no treatment was given, and, according to Marsden, none of his patients developed fits but in only three was there an appreciable fall in blood-pressure after treatment. Smith & Smith [1938] are of the opinion that in toxæmias of pregnancy (eclampsia and pre-eclampsia) there is primarily an excess of 'prolan'¹ but also a deficiency of progesterone resulting in a reduced conversion of oestrone to oestriol and consequently a greater destruction of all the oestrogens. Such cases were treated with large amounts of progesterone and oestradiol benzoate but showed no clinical improvement.

Savage & Wylie [1937] and Savage, Wylie & Douglas [1938] contrasted a series of toxæmic patients having chronic nephritis or pre-eclampsia with a group of normals and demonstrated a definite lowering in the levels of oestrogen excreted in late pregnancy; oestrogen therapy, however, caused no clinical improvement. An oestrogen-prolan imbalance has been postulated by Smith & Smith [1937]; Nicol [1938] attributes eclampsia to an increase in the amount of oestrogen in the blood.

It is clear from the data obtained in the case of patient No. 67 that

¹ Dr. Kapeller-Adler's negative histidine finding in the eclamptic case here described suggests that a deficiency of 'prolan' existed.

eclampsia cannot be ascribed to a lack either of progesterone or of oestrogen or of both, although it may often be found in such circumstances, and that some other factor, common to all, must be responsible. Since we are concerned with ascertaining only whether an hormonal imbalance exists in such conditions it is not intended to discuss the part possibly played by placental infarction [Bartholomew & Colvin, 1938], hypertensive arterial disease [Dieckmann & Brown, 1938], the custom of salting food [de Snoo, 1937], or the increased effects of the posterior pituitary-adrenal system due to lack of neutralization of these hormones in the liver [Hofbauer, 1937].

Case 68 has been difficult to assess accurately owing to the loss of urine due to incontinence (the patient suffered from pyelitis and disseminated sclerosis). The allowances made for such losses were based on the estimates provided by the nurse-in-charge. The birth was premature by about 8 weeks (weight at birth 5 lb. 3½ oz.), but the infant was successfully reared. There was severe pyelitis and albumen was present in the urine, this was ++ at 5 days antepartum, + at 3 days, a trace during the 2 subsequent days, and for the 4 days postpartum again +. Her temperature was 102° and pulse 104 from the 6th to the third days antepartum when the temperature fell to 100·6°; before labour there was another drop to 99° and thereafter it was steady at 97·2°. Her highest blood-pressure during the period was 132/86.

The hormone output for the short time covered was low in all the forms analysed, but especially in combined oestrone. Two items are of interest: the rise in combined oestrone during labour—a rise which is doubtful owing to the difficulty of computing a 24 hours' output on a single 4 hours' collection; also the fairly high excretion of pregnanediol in the 24 hours immediately postpartum in relation to the output prior to labour.

Case 60, the last of the group showing abnormal features, was excreting both oestrogen and pregnanediol at a fairly high rate up to the commencement of labour (Table VI). The notable feature in this case, however, is the amount of hormone excreted 2 days after delivery: 19 mg. pregnanediol and 2·6 mg. combined oestriol. The extract was exhausted before the unit of combined oestrone was reached. Even at 5 days postpartum pregnanediol was recovered (1,720 ml. urine used). It is possible that this slow hormone elimination was partly responsible for the mastitis which developed, as generally by the second day no traces of oestriol are found.

Hormone values in the puerperium

Postpartum urines were examined in order to ascertain whether the systemic changes associated with the establishment of lactation, par-

ticularly the engorgement of the breasts which occurs about the 5th day, were accompanied or preceded by hormone excretion. The appended list of values (Table VII) shows that a considerable amount of pregnanediol was recoverable during the 24-hour period immediately following delivery, more especially when the first urine passed was included. The values varied greatly, depending largely on the rate of excretion at the time of labour, viz. 38, 16, 17, 58, 25, 3 and 6 mg. A comparison with the amounts excreted before parturition shows that generally a drop to a half or a third of the antepartum value of pregnanediol occurred at parturition. Numerous instances can be found on the charts of drops of equal or greater magnitude towards the end of pregnancy without labour changes ensuing. In two instances the postpartum values are only a little below those at the onset of labour: patients 64 and 68 excreted 16 and 25 mg. compared with 25 and 38 mg. before labour; in case 64, however, it is probable that a low urinary output was associated with faulty hormone elimination. In one of the stillbirths (case 66), no trace of pregnanediol was found in the postpartum specimen, which is not surprising when one considers the length of time that the child was dead. Patient 67 (another stillbirth), on the other hand, has a drop of only 60% of her previous high excretion and passes after delivery as much as some excrete at their maximum (58 mg.). The third stillbirth experienced a sudden and very marked drop from 104 mg. to 6 mg., similar to that in oestriol though less marked even than this (11.3 mg. to 0.3 mg.). On the whole, the elimination of pregnanediol at parturition was proportionately less than that of oestriol, yet all traces of pregnanediol had usually disappeared by the second day after delivery, whereas 5 and 8 μ g. of combined oestrone were being excreted 4 days postpartum. The possible connexion between the slow hormone elimination and the occurrence of mastitis in case 60, in whom the values were still high 2 days postpartum, has already been discussed. On the 4th day after parturition Wilson, Randall & Osterberg [1939] recovered a mean value of 6.0 mg. pregnanediol in 18% of their cases, but in the remainder negative or minimal amounts were obtained. Table VII shows that at 4 days postpartum combined oestrone was being excreted in amounts ranging from 5 to 13 μ g. That this hormone was not responsible for the breast development normally occurring about the 5th day is indicated by the fact that in each of the three stillbirths no breast enlargement occurred although the oestrogen levels were similar to those found in normal cases.

In order that a clearer idea may be obtained of the relation between the combined oestrogens in the same sample of urine, Table VIII gives the equivalent of urine used for assay on mice in the cases of three normal and two abnormal patients. The marked rise in hormone excretion which

occurred in case 48 at the fifth month is evident; also that there is little change in the amounts of urine used during the last week of gestation.

DISCUSSION

It has been the author's endeavour to present data regarding the hormone excretion in pregnant women approaching labour which would throw some light on the changes which take place at parturition, in the hope that it might be possible to say (a) that at so many days after a specified change parturition is sure to occur, and (b) that labour occurs when the hormone relationship or concentration is of a certain nature.

Two obvious defects occurring in the investigation have to be accepted before an evaluation of the data can be made, viz. the absence of information regarding the amount of *free* oestrogen excreted at the end of pregnancy, and also of the amount of gonadotrophic hormone. Analyses of the former are in hand and the relationship between the various oestrogens and pregnanediol in the same specimen of urine is being ascertained. The second drawback to any hormone analysis involving the oestrogens is the fact that not only is an unknown proportion of the oestrogens formed in the body unrecoverable by present methods but also augmenters exist which may play an important part in determining their physiological potencies [Emmens, 1939; Callow, Callow, Emmens & Stroud, 1939]. The data do, however, provide valuable information, not hitherto available, as to the relationship between pregnanediol and the combined oestrogens in some cases of normal and of abnormal gestation, using the same methods of analysis. In interpreting these data the reader is reminded that the face values of combined oestriol should probably be increased by 40–50%.

It is evident that great differences exist between individuals (and in the same individual) not only in the amount of hormone excreted but in the relationship between the three substances—combined oestrone, combined oestriol and pregnanediol. Generally speaking, a high excretion of pregnanediol is associated with a high excretion also of combined oestrone and is reflected in the level of combined oestriol, but there is no direct relationship in point of time, as the following ratios of combined oestrone to pregnanediol show (expressed in mg. recovered)—5.3 : 127 (case 61); 3 : 60 (case 48); 2.4 : 60 and 2.2 : 130 (both in case 65). The colossal output of pregnanediol in cases 67 and 62 a little before parturition seems to have borne only a slight relationship to the oestrogen excretion, which, in the latter, was falling during the whole period of the rise in pregnanediol. The absence of a quantitative relationship between the three hormones is surprising in view of the calculations of an oestrogen/progesterone

ratio arrived at in experimental animals [Courrier, 1935; Robson, 1936], the castrate monkey [Hisaw, 1935; Engle, Smith & Schelesnyak, 1935; Zuckerman, 1937; Corner, 1938; Engle & Smith, 1938; Hisaw & Greep, 1938] and the human castrate [Kaufmann, 1935; Elden, 1938]. Corner [1938] estimates that in the prevention of endometrial disintegration, 1 mg. of progesterone is physiologically equivalent to 200 to 300 I.U. of oestrone. If the record of case 48 is examined on this standard, and allowance is made for the fact that only 42-70% of the progesterone secreted is recoverable in the urine as sodium pregnanediol glucuronide [Venning & Browne, 1938], the balance is considerably in favour of progesterone until the 250th day, after which the ratio above mentioned is attained and frequently passed in favour of oestrogen.

It required *daily* hormone analyses (as in the case of patient 48) to reveal what may be the nature of the normal excretion of hormones at the approach of parturition, viz. a rhythmic rise and fall. If this be borne in mind it becomes less perplexing when one observes that in spite of a marked fall in the level of oestrogen seen in several of the charts, labour does not ensue. Robson [1939], as the result of experiments on rabbits, advocates the oestrogen-deprivation theory of parturition, others that of progesterone-deprivation. The frequent occurrence of a peak of excretion of pregnanediol and/or of oestrogen during labour inclines one to the opinion that the body, during the last fortnight and especially during the last 8 or 10 days of gestation (i.e. covering the 'peak' period), is engaged in a process of lowering the hormone level by means of hormone elimination and it is immaterial, therefore, whether labour takes place when the rate of excretion is high or low. Furthermore, it has been shown that, in terms of values, the drop in pregnanediol which occurs at parturition is frequently of less magnitude than occurs on a single day during the last 2 weeks of gestation, and, also, considerable amounts of the glucuronide are recoverable immediately after delivery. The same applies to the excretion of the combined oestrogens. It is clear, therefore, that, in the human subject, a marked lowering in the level of the combined oestrogens and/or of progesterone is insufficient to initiate the labour changes, and that the hormone concentration is never such that one can envisage the exact day of parturition.

Since the secretion of the oestrogens and progesterone in the non-pregnant animal is commonly held to be under the control of the anterior pituitary, the obvious conclusion from the data presented is that the fall in concentration of both of these hormones is due to a decrease in the gonadotrophic secretion of the pituitary. Such a theory in explanation of parturition was recently put forward by Robson [1940] and is based mainly on the fact that pregnancy is terminated in the rabbit 24-48 hours after

hypophysectomy but can be maintained for 5 or 6 days after removal of the hypophysis if oestrogen or progesterone is administered. That this was not adequate replacement therapy is shown by the fact that, with a single exception, all the foetuses were born dead. The manifest rhythm observed in the falling excretion of oestrogen and pregnanediol, markedly demonstrated in case 48, though conceivably controlled by waning hypophyseal activity, suggests rather a positive stimulus. Furthermore, although a diminished gonadotrophic secretion is clearly demonstrable at the end of gestation in that the Aschheim-Zondek test for pregnancy is frequently negative after the 7th month, the fact that this is true so long before term tends to militate against Robson's theory. Moreover it has been shown by Kennedy [1933] that the concentration of gonadotrophic hormone in the *blood* reaches its maximum during the 32nd-40th weeks in human pregnancy, and follows in the main, the curve of oestrogen and pregnanediol excretion.

It may not be amiss to ask when labour changes can be said to commence. Probably 21-28 days before parturition; in short, they date from the commencement of the 'peak' period in combined oestrogens and pregnanediol, a period characterized by a gradual but marked rise to a maximum excretion reached about 9-5 days before parturition, followed by gradual fall. It is during the latter time that, according to Cohen *et al.* [1935] there occurs a rise in the free forms of oestrogen—a change which, they suggest, may prove to be an important factor in the initiation of labour. In these studies our concern has been with the initial changes rather than with the culminating act. The hormone disturbance which takes place during the last three weeks of gestation may denote an increased hormone secretion by the placenta; the object of the rise in combined oestrone being to antagonize or reduce luteal activity (progesterone), i.e. to arrest the action of the hormone which, up to this stage, has been mainly responsible for uterine quiescence, and, therefore, for the maintenance of the pregnant condition.

However, since there is a simultaneous increase in progesterone output (as determined by the pregnanediol glucuronidate recovered) the alternative possibility presents itself, viz. that this period of peak excretion is mainly a period of elimination, the purpose of which is to lower within the body the concentration of all those hormones that are intimately concerned with the maintenance of the pregnant condition and to which the following functions have been attributed by various authors:

- (a) The production (by oestrogen) of hyperaemia in the uterus, as the result of which there is a significant rise in its metabolic activity as well as an augmentation of uterine growth [Barcroft & Rothschild, 1932; Markee, 1932; Neumann, 1934; Buchheim & Zaleski, 1930;

Courrier & Bouin, 1929] with resultant activation of the uterine muscle [Reynolds, 1938; Reynolds & Foster, 1939].

- (b) The suppression of the follicle-stimulating hormone of the pituitary by oestrogen, a function which is generally accepted. There is evidence to show that oestrogen also promotes the release of the luteinizing hormone in some species [Hohlweg, 1934; Fevold, Hisaw & Greep, 1936] whereas progesterone inhibits its release and also prevents ovulation [Makepeace, Weinstein & Friedman, 1937; Dempsey, 1937; Astwood & Fevold, 1939].
- (c) The inhibitory effect of progesterone upon uterine reactivity to oxytocic substances as observed in some species [Knaus, 1929; Robson & Illingworth, 1931] but not confirmed by others in the human subject.

From the above it would seem that, on the positive side, we have a hyperaemic uterus greatly augmented in growth and with its metabolism raised and its myometrium well developed; on the negative side, pituitary activity is suppressed and, possibly, uterine motility also. In spite of this, it is known that the concentration of luteinizing hormone in the blood increases during pregnancy [Kennedy, 1933], although that in the urine decreases. Apart from the primary function of these hormones, described above, the purpose of so great a concentration prior to parturition is not clear. The process of hormone elimination apparently reaches a maximum a few days before labour and, allowing for the fact that the hormone content of the urine must lag a little behind the initial processes giving rise to it, there is time for other factors to come into play which precipitate the act of labour. The data have shown that in the process of hormone elimination the kidneys play an important part. Other factors, such as the mechanism for the conjugation of oestrogenic glucuronides in the liver and, possibly, elsewhere also, must function on an increased scale. It may well be that the increased hormone elimination occurring prior to labour is directly connected with a lowering of the blood-supply in the uterus, the withdrawal of the hyperaemia-producing oestrogen causing a partial anaemia [Barcroft & Rothschild, 1932]. If one accepts the theory that luteal activity is maintained by oestrogen [as demonstrated by Allen, 1937; Heekel & Allen, 1939; Robson, 1939; and others] a further need for lowering the level of oestrogen presents itself; yet it is not clear that a fall in oestrogen precedes the fall in progesterone. It seems likely that the hormone-deprivation theory (either in the terms set forth by Robson [1939] or by Corner [1938] in explanation of menstruation) is hardly adequate to cover all the processes involved in the onset of labour.

The data have been examined in order to ascertain whether the length

of the labour process could be related in any way to the values for combined oestrone and pregnanediol during the 'peak' period. The shortest labour—2 hours—was experienced by case 67, a severe eclamptic who gave birth to a stillborn child and whose maximum excretion of combined oestrone in 24 hours was 3.3 mg. In two patients with almost the same output (2.9 and 3.0 mg.) labour lasted 7 and 17 hours respectively, and still higher values—4.2 and 5.3 mg. were associated with labour lasting 15¹⁰₀₀ and 10 hours. It is obvious, therefore, that a high oestrogen excretion during the 'peak' period does not ensure a short labour, and it is of interest that the patient with pyelitis (case 68) in whom the level of combined oestrogen was low (maximum 0.5 mg.) had a short labour of only 4½ hours.

A similar lack of correlation is observable between the length of labour and the rate of excretion of pregnanediol during the peak period, as the following figures show: 274 mg. pregnanediol—2 hours labour; 123 mg.—15¹⁰₀₀ hours; 104 mg.—27⁵⁰₀₀ hours; 127 mg.—10 hours; 42 mg.—4½ hours; 42 mg.—14 hours.

Reference has been made to the apparent existence of a rhythmic rise and fall in hormone excretion during the last 3–4 weeks of gestation. It may well be that the stimulus controlling this rhythm is that which is responsible for initiating labour and parturition.

Comparison of the data for normal pregnancy and for toxæmias provides no evidence of the existence of a 'hormone imbalance' in the latter—a phrase too loosely used, seeing that the balance in normal gestation is so variable. An abnormal outcome of pregnancy may be associated equally with low and with high hormone values, and the figures for a severe eclamptic given in this report clearly demonstrate that a high oestrogen and pregnanediol excretion may characterize the condition in certain cases. The necessity for the accumulation of consecutive data in a large number of normal pregnancies and toxæmias is obvious.

In the course of this investigation, which has covered a period of 2 years, improved methods of extraction and separation of the urinary oestrogens have been reported [Callow *et al.*, 1939; and Smith, Smith & Schiller, 1939]. In order that the data might be of value for purposes of comparison no change in the method was made; also, on the further series in hand the same method has been employed.

SUMMARY

1. The excretion of the combined oestrogens and of pregnanediol has been examined in women during the last 2–3 weeks of pregnancy, labour and the puerperium, in the hope of establishing a normal relationship

between the excretion of these substances with which abnormal pregnancies and cases of toxæmia might be compared. At the same time the hormone changes associated with labour, parturition, and the establishment of lactation were examined.

2. The series comprises normal pregnancies and toxæmias, and also one normal woman followed throughout the whole course of pregnancy.

3. No definite quantitative relationship between the hormones could be established, although a general similarity in the outline of the curves, but without coincidence, was observed in normal cases.

4. During the last 3 weeks of gestation a marked increase in the excretion of combined oestrogens and of pregnanediol takes place, the maximum excretion being attained generally 8-10 days before parturition.

5. Daily hormone analyses indicate a rhythmic rise and fall in oestrone, oestriol and pregnanediol values prior to labour and the controlling stimulus of this rhythm may be responsible for the onset of labour.

6. The purpose served by the high excretion of hormones prior to labour is discussed as also the relation between labour and hormone elimination, oestrogen-deprivation and progesterone-deprivation.

7. Frequently the kidney played an important part in lowering the level of hormone-concentration in the body by furthering elimination.

8. No connexion exists between the amount of combined oestrogen and pregnanediol excreted during the 'peak' period and the duration of labour.

9. It was impossible from hormone analyses to forecast the day on which labour would commence, as it might occur equally at the top and at the bottom of a rhythmic rise and fall in hormone excretion.

10. In the group of toxæmias high values for oestrone and very high values for pregnanediol were found in a severe pre-eclamptic; and a sudden marked rise in pregnanediol followed intra-uterine death in another patient.

11. The need for data of relative hormone values taken at frequent intervals in large numbers of normal and abnormal gestations is pointed out.

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Table II. *Excretion of oestrogens per 24 hours at the end of gestation*

Case no.	Days before parturition	Combined oestrone mg.	Oestrogen in untreated urine m.u.	Combined oestriol mg.	Amount of urine in 24 hours ml.	Clinical details, &c.
50	4	0.81	3240	—	1350	
51	15	0.85*	2926	—	1570	Low H.F. forceps delivery; commencing secondary uterine inertia; labour
(Fig. 1)	12	0.70†	2550†	6.8	1700	15 5/60 hr.; episiotomy; full-time child, 8 lb.
	8	0.70	3117	12.0*	1700	Up to time of labour.
	5	0.39	3060	4.25	1700	
	1	0.42*	4650*	3.6*	1550	
52	20	0.46*	1500*	—	1710	S.D.; labour 8 hr.; full-time child, 7 lb. 9 oz.
(Fig. 2)	17	0.65	1320	4.5*	1760	
	13	0.82	1317	4.6	1580	
	10	0.76	1433	5.9	1720	
	6	1.10*	2717	8.2	1630	
	3	0.38	1960	8.0*	1400	False labour after collection of this specimen.
53	19	0.45	1760	—	1320	S.D.; full-time.
(Fig. 3)	15	0.71	1622	5.6	1390	
	12	0.79	3025	6.6	1650	
	8	1.43*	2900	5.0	1740	
	5	0.90	4676	5.7*	1670	
	1	0.45*	4150	6.6*	1660	Up to 3 hours before labour.
54	1	0.61	3700	5.03*	1480	Parturition on day after collection of specimen.

55 (Fig. 4)	33	0.13*	2133	4.3	1600	S.D.; labour 12½ hr.; full-time child, 7 lb. 13 oz.
	30	0.16	3100	5.8	1700	
	26	0.70*	3700	6.3	1700	
	23	0.90*	2900	11.4	1670	
	19	0.86	2250	10.1	1690	
	16	0.84	3025	13.2	1650	
	12	1.62*	1972	15.5	1690	
	9	1.90	2450	13.0*	1750	
	5	1.03	2032	6.8	1710	
	2	1.13	2704	15.6*	1690	
56	6	0.73	2998	9.5	1660	S.D.; labour 9 hr.; full-time child, 7 lb. 8 oz.
	3	0.75	4280	9.23	1710	
57 (Fig. 5)	22	0.95*	2400	6.5	1750	High blood pressure. S.D.; labour 8 hr.; full-time; 7 lb. 11 oz.
	18	0.83*	3010	17.2†	1720	
	15	1.46*	2910	6.3*	1280	
	11	0.91	4480	24.9	1680	
	8	1.60*	2180	15.5	1550	
	4	1.20	1500	17.0*	1250	
	1	0.95	1620	13.0†	1080	
58 (Fig. 6)	12	0.91*	3360	4.8*	1680	S.D.; labour 13 55/60 hr.; full-time male 8 lb. 5 oz.; mild pre-eclampsia, oedema of ankles, B.P. 180/120 dropped to 140/80 before delivery; slight albumin. Up to time of labour.
	8	1.40*	1420	9.7	1700	
	5	0.75	1700	13.6	1700	
	1	0.83	1376	4.9	1720	

* = 40 mico used.
† = 80 mico used.

‡ = 100 mico used.

S.D. = Spontaneous delivery.

a.p. = antepartum.

p.p. = postpartum.

These symbols are used throughout the subsequent tables.

Table III. *Daily excretion of oestrogens and pregnanediol throughout gestation.*

Case 48. (Figs. 7 and 7a)

Days pregnant	Combined oestrone mg.	Combined oestriol mg.	Pregnanediol mg.	Amount of urine per 24 hr. ml.	Remarks
48	0.04†	0.025†	—	1470	No abnormal symptoms throughout gestation and lactation; S.D.
75	0.012*	0.01	—	1343	
103	0.04*	0.09*	—	1627	
131	0.01*	0.5†	—	1487	
159	0.3†	2.7*	21	1677	
187	0.3	3.2	30	1450	
215	0.36	3.7	41	1480	
243	0.82	7.5	60	1605	
250	0.86	11.0	55	1597	
257	1.4	11.0	40	1370	
264	2.8	20.0	55	2300	
267	2.0	12.0	57	1355	
270	1.6	15.7	62	1306	
271	1.3	8.4	51	1205	
272	—	—	46	1650	
273	2.8	8.6	43	1715	
274	3.0	17.3	60	2030	
275	1.3	9.8	40	980	In spite of low urinary output, excretion remained stationary.
276	1.3	8.1	48	805	
277	1.9	7.0	40	1090	
278	1.6	14.6	30	1460	
279	0.75	—	25	1280	
280	1.1	11.7	42	1830	
281	0.2	—	20	664	12-hr. specimen up to labour, calcd. on 24-hr. basis.
282	1.2	3.4	6	1271	Labour 17-hr., calcd. on 24-hr. basis.
postpartum	0.9	1.1	3	2770	

* † &c. as in Table II.

Table IV. *Daily excretion of oestrogens and pregnanediol: 4 normal women at end of gestation*

Case no.	Days antepartum	Combined oestrone	Combined oestriol	Pregnanediol	Amount of urino per 24 hr.	Clinical details
61 (Fig. 4)	37	mg. 0.5	mg. 0.7	mg. 60	ml. 1740	S.D.; labour 10 hr.; full-time male 7 lb. 6 oz.; puerperium uneventful until ovo of 4th day when pulse rose to 120, temp. 99°; some delay in involution of uterus, growth of <i>B. coli</i> obtained on 7th day; condition cleared up by 10th day p.p.
	33	0.8	1.4	71	1720	
	29	0.4	9.5†	66	1730	
	26	1.2	19.8†	90	1420	
	22	1.9	14.8	83	1750	
	16	2.3	12.2	101	1740	
	12	4.8	19.6	112	1780	
	9	4.7†	21.2	116	1764	
	5	6.3*	24.6	127	2580	
	2	1.9	19.3†	82	1135	
	1 day p.p.	0.52	8.0*	38	3495	
	4 days "	0.005*	0.08†	nil	1160	
65 (Fig. 5)	7	2.1	9.7	7	974	S.D.; labour 7 hr.; full-time female 7 lb. 3 oz.; some engorgement of breast on 4th-5th days. Up to labour—18-hr. specimen, calcd. on 24-hr. basis; parturition 1½ hr. later.
	6	2.2†	17.2	130	2020	
	5	1.5	13.4*	100	1410	
	4	—	—	58	744	
	3	1.6	13.3	60	2650	
	2	1.4*	0.5*	41	2730	
	1	2.9*	7.4	61	1747	
	1 day p.p.	0.2*	1.0	17	1530	
	4 days "	0.013	—	nil	1280	
	1	4.2†	8.75	112	1250	
	9 hr.	—	9.3	123	984	
	2 days p.p.	0.06	—	nil	780	
64 (Fig. 10)	4	0.63†	10.4	36	1730	S.D.; labour 14 hr.; full-time male 7 lb. 5½ oz.; episiotomy; treated for varicose veins in legs and thigh one month before delivery. 12-hr. collection, up to time of labour; parturition 12 hr. later.
	3	—	—	42	680	
	2	0.85†	4.2	28	835	
	1	1.05‡	0.8	25	1140	
	1 day p.p.	1.0‡	1.0*	16	1900	
	4 days "	0.005†	—	nil	2000	

* † &c. as in Table II.

Table VI. *Daily excretion of oestrogens and pregnanediol in 5 women having complications at the end of gestation*

Case no.	Days antepartum	Combined oestrone mg.	Combined oestriol mg.	Pregnanediol mg.	Amount of urine per 24 hr.	Clinical details
66	4	0.05†	0.8*	20	ml.	Intra-uterine death (male); 3-4 weeks premature; macerated—no foetal movements felt for last 3 weeks; foetal death confirmed by X-ray 1 week before induction; membranes ruptured by direct puncture; 10,000 units Progynon given 4 times; no p.p. swelling of breasts; slight oedema of ankles during 5th month.
(Fig. 11)	3	0.06†	0.8*	13	1490	
	2	0.06	0.6	15	1200	
	1	0.05*	0.3	11	2050	
	1 day p.p.	0.02	0.1	nil	860	
					1940	
62	29	0.77*	—	—	1740	Intra-uterine death; no foetal heart felt on admission; full-time (acc. to dates), 5 lb. 6 oz.; labour 27 50/60 hr.; shocked, responding; pulse 128, fell to 110 after delivery of placenta, free 3rd stage. No toxic symptoms; difficulty in emptying bladder during first few days p.p.
(Fig. 12)	25	0.83	7.5†	28	1735	
	21	1.0	10.5	33	1680	
	18	1.7	9.0	35	1460	
	14	1.3	11.0	38	1710	
	10	1.6	9.5	34	1720	
	8	1.0	6.5	70	1740	
	4	0.8*	6.5	46	1380	
	1	0.7	11.3	104	2440	{ 12 hr. specimen up to time of labour; parturition 20 hr. later.
	1 day p.p.	0.3	0.3	6	820	
	3 days p.p.	0.007	0.05	nil	860	
	6 "	0.002	—	—	500	
67	13	2.6	—	96.75	2420	Still-birth; full-time child 10 lb. 8 oz.; labour 2 hr.; pituitrin given at 2nd stage. Severe pre-eclampsia, stoutness, albuminuria ++++, high blood-pressure: B.P. 172/96 at 15 days a.p. did not fall till 3 days a.p.; no p.p. swelling of breasts (see text). Para 3, ago 33.
(Fig. 13)	11	2.4*	13.8*	158.5	1250	
	9	1.5	5.0*	132.6	1470	
	6	3.1*	7.6	116.4	1900	
	3	3.3†	12.0	274.0	1720	

	1	—	134.5	1050	Up to time of labour; single passage of urine multiplied by 7 for 24 hr. estimation.
	During labour	2.3*	Centrifuge cup broke	1925	275 ml. in 2 hr.; × 7 = 1925 ml. in 24 hr.
	1 day p.p.	0.4	58	1800	
	5 days p.p.	0.008	—	1850	
64	6	0.5	42	1850	S.D.; 7-8 weeks premature; 5 lb. 3½ oz.; labour 4½ hr.; severe pyelitis, also disseminated sclerositis; temp. 102°-100.6° for 4 days; 99° 1 day a.p. Albumen present; highest B.P. was 132/86.
	4	0.4*	38	1680	
	1	0.2	38	1440	
	During labour (4 hr.)	0.35	—	2340	390 ml. in 4 hr.; × 6 = 2340 ml. in 24 hr.
	1 day p.p.	0.08*	25	1420	
60	1	1.26*	53	1700	14 hr. before and 10 hr. during labour.
	2 days p.p.	1*	10	1500	S.D.; full-time child 8 lb. 5 oz.; labour 21 20/60 hr.; influenza cold during puerperium, mastitis of left breast; temp. 100°-101° 16th-19th days after delivery.
	5	—	nil	2220	

Table VII. *Hormone values in the puerperium*

Case no.	Days postpartum	Combined oestrone	Combined oestriol	Pregnanediol
		mg. 0.9	mg. 1.1	mg. 3
48	1 ¹			
60	2	unit not reached	2.6	19
	5	—	0.4	nil
61	1	0.52	8.0	38
	4	0.005	0.08	nil
62	1	0.3	0.3	6
	3	0.007	0.05	nil
	6	0.002	—	—
63	2	0.06	nil	nil
64	1	1.0	1.9	16
	4	0.005	—	nil
65	1	0.2	1.0	17
	4	0.013	—	nil
66	1	0.02	0.1	nil
67	1	0.4	1.9	58
	5	0.008	—	—
68	1	0.08	0.31	25

¹ First urine passed after delivery was discarded.

Table VIII. *Equivalents of urine used to obtain oestrogenic effect (combined oestrone and combined oestriol) at different stages of gestation*

Case no.	Stage of pregnancy	For combined oestrone	For combined oestriol	Remarks
		ml.	ml.	
48	1st month	2.0	35.0	
	2nd "	5.0	30.0	
	3rd "	4.0	15.0	
	4th "	3.0	2.0	
	5th "	0.5	0.5	
	6th "	0.5	0.5	
	7th "	0.4	0.4	
	8th "	0.2	0.15	
	9th "	0.2	0.15	
	1 week later	0.1	0.1	
	" "	0.05	0.05	
	2 weeks later	0.1	0.05	
	Day before labour	0.15	0.15	
61	37 days antepartum	0.4	1.0	
	29 " "	0.2	0.2	
	22 " "	0.1	0.1	
	16 " "	0.05	0.1	
	9 " "	0.025	0.05	
	2 " "	0.05	0.025	
	1 day postpartum	0.4	0.4	
	4 days "	10.0	10.0	
65	7 days a.p.	0.04	0.1	
	6 "	0.1	0.1	
	2 "	0.2	0.4	
	1 "	0.05	0.2	{ Up to time of labour, parturition 1½ hr. later.
	1 day p.p.	1.0	1.5	
	4 "	10.0	—	
62	25 days a.p.	0.2	0.2	
	18 "	0.05	0.1	
	10 "	0.1	0.2	
	4 "	0.2	0.2	
	1 "	0.2	0.2	
	1 day p.p.	0.4	3.0	
	3 "	10.0	10.0	
67	11 days a.p.	0.05	0.1	
	6 "	0.05	0.25	
	3 "	0.05	0.1	
	Labour	0.05	—	
	1 day p.p.	0.5	1.0	
	5 "	12.0	—	

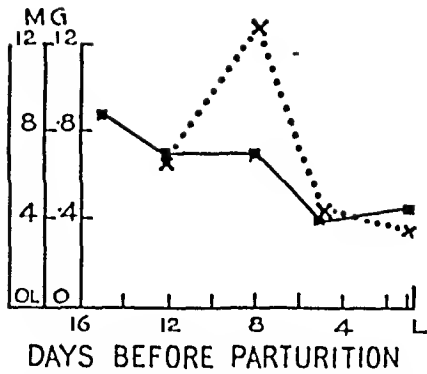


FIG. 1. (Caso 51, Table II) X...X = combined oestriol (OL), ■—■ = combined oestrone (O), L = labour.

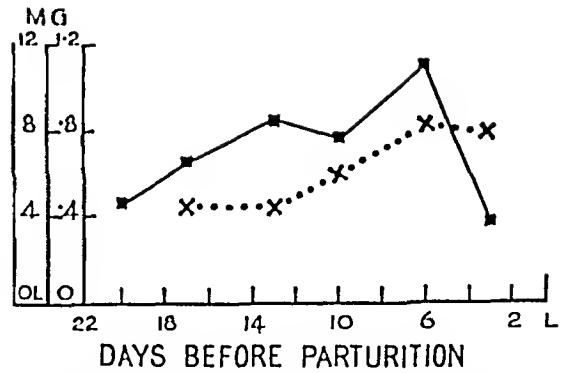


FIG. 2. (Caso 52, Table II) X...X = combined oestriol (OL), ■—■ = combined oestrone (O), L = labour.

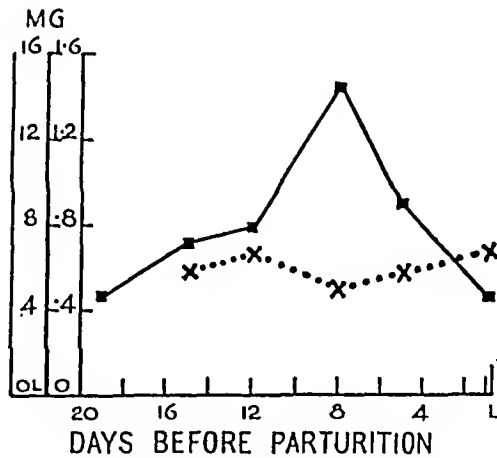


FIG. 3. (Caso 53, Table II) X...X = combined oestriol (OL), ■—■ = combined oestrone (O), L = labour.

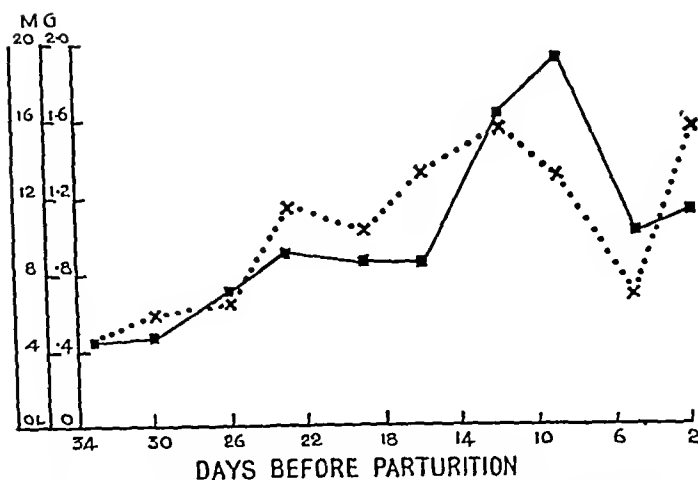


FIG. 4. (Case 55, Table II) X...X = combined oestriol (OL), ■—■ = combined oestrone (O).

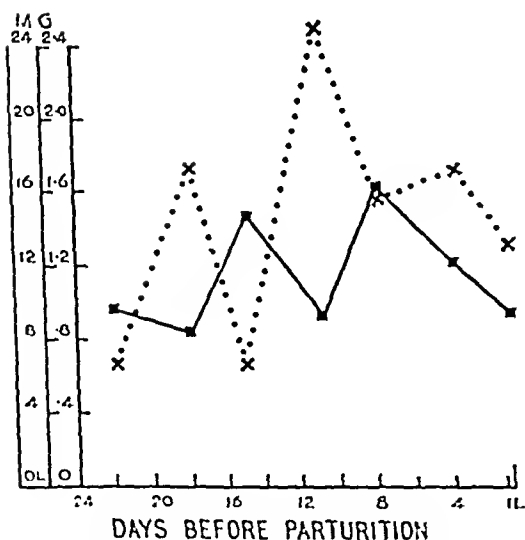


FIG. 5. (Case 57, Table II) X...X = combined oestriol (OL), ■—■ = combined oestrone (O), L = labour.

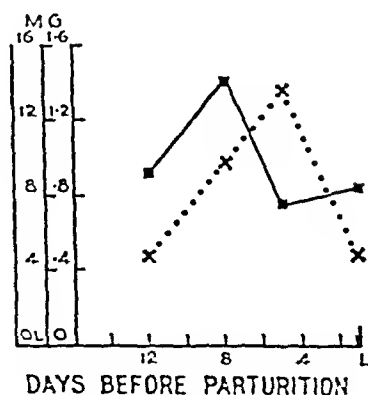


FIG. 6. (Case 58, Table II) X...X = combined oestriol (OL), ■—■ = combined oestrone (O), L = labour.

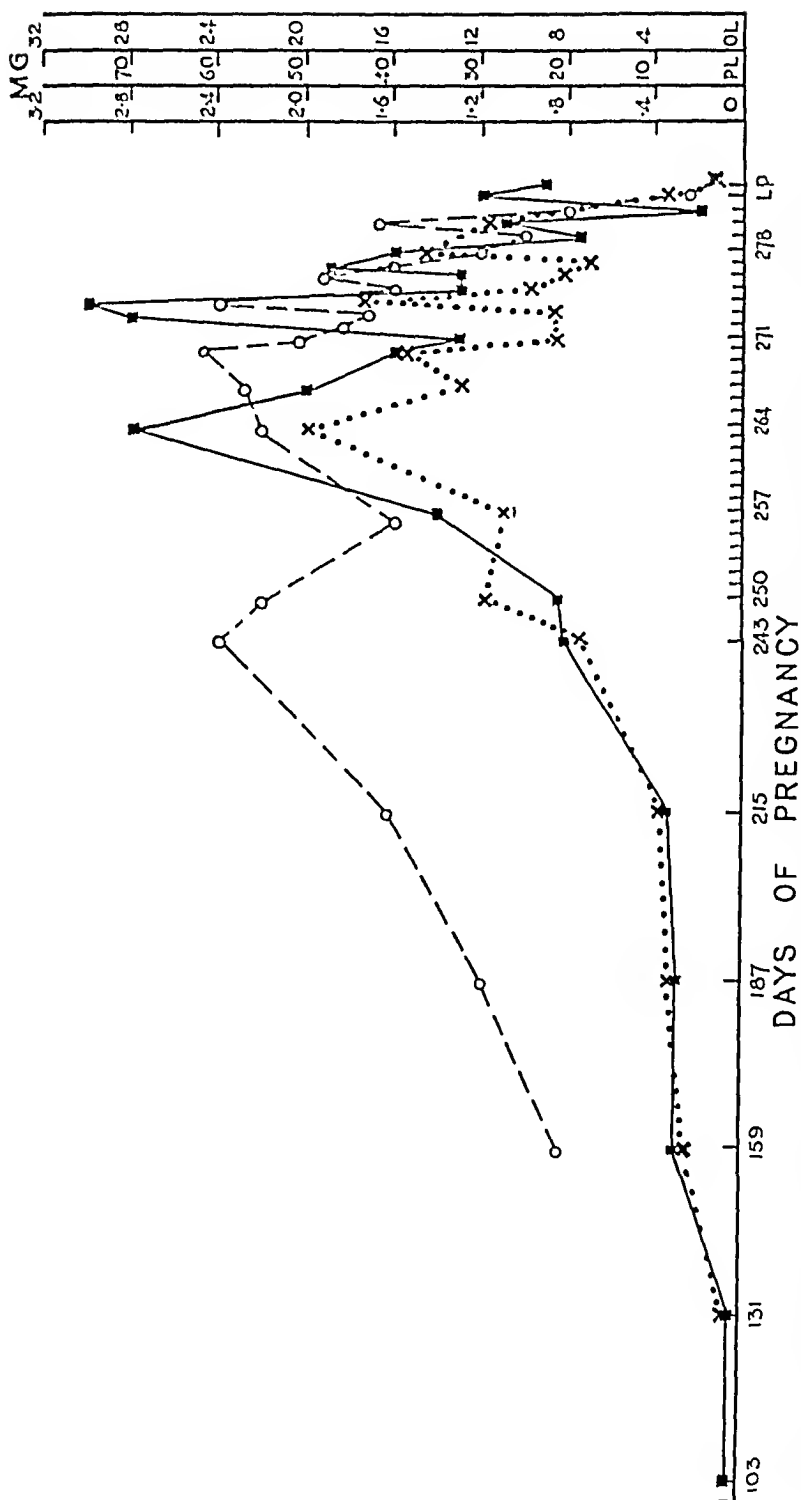


Fig. 7. (Case 48, Table III) X...X = combined oestriol (OL), ■---■ = combined oestrone (O), O---O = pregnanediol (PL), L = labour and P = parturition. To conserve space the first 3 months of pregnancy are omitted, being given in Table III. The last month of pregnancy is drawn on a time-scale double that of the earlier months. See also Fig. 7a.

M G

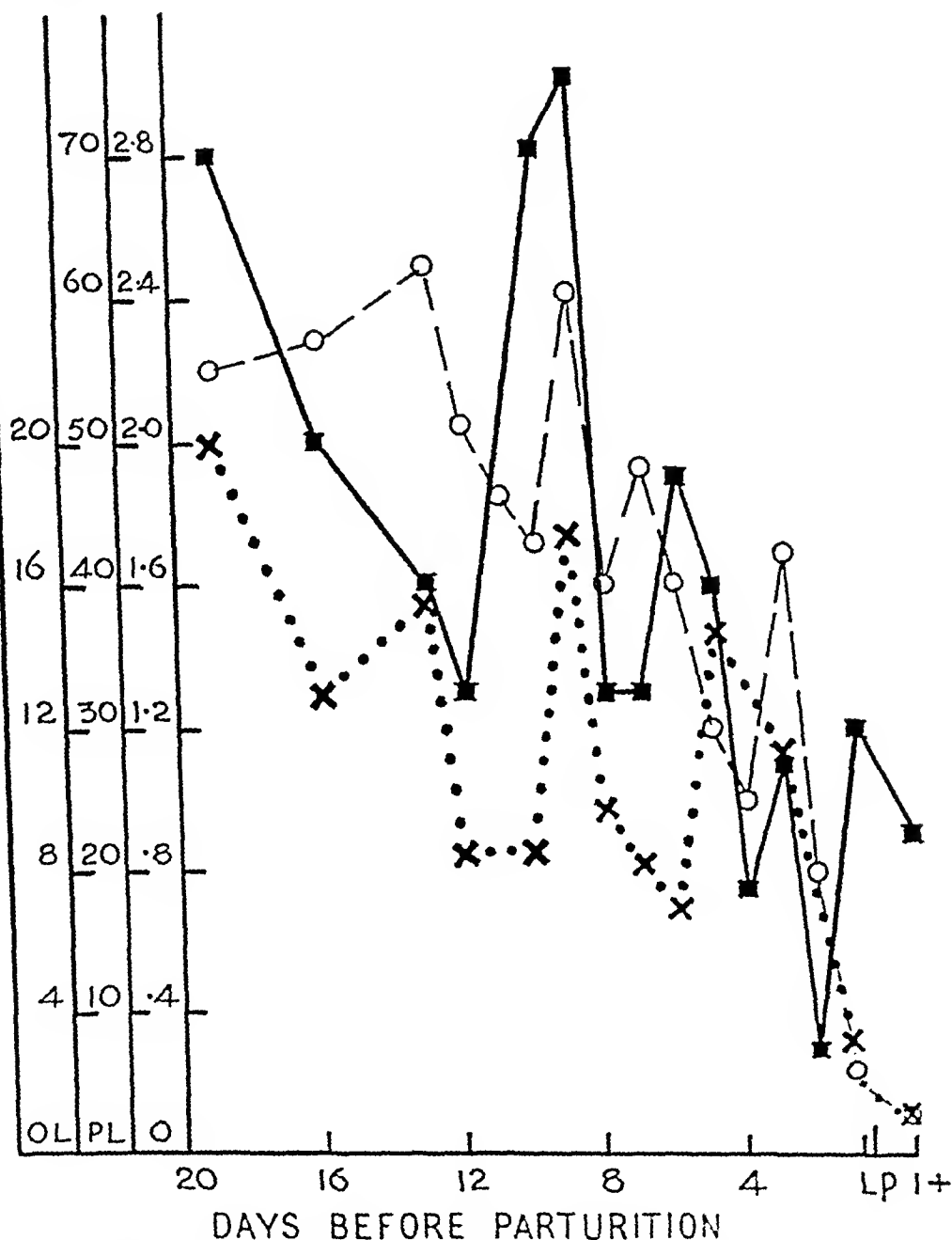


FIG. 7a. (Case 48, Table III) X...X... combined oestrol (OL), ■—■... combined oestrone (O) and O---O... pregnanediol (PL), L = labour and P = parturition.

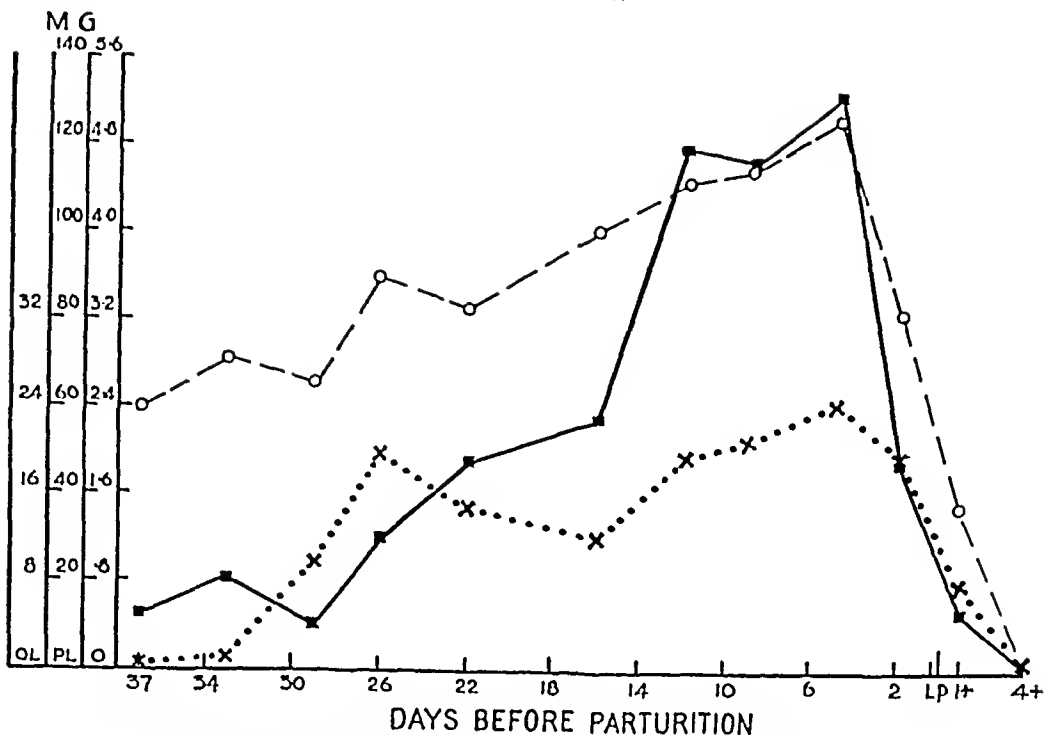


FIG. 8. (Case 61, Table IV) X...X = combined oestriol (OL), ■—■ = combined oestrone (O) and O---O = pregnanediol (PL), L = labour and P = parturition.

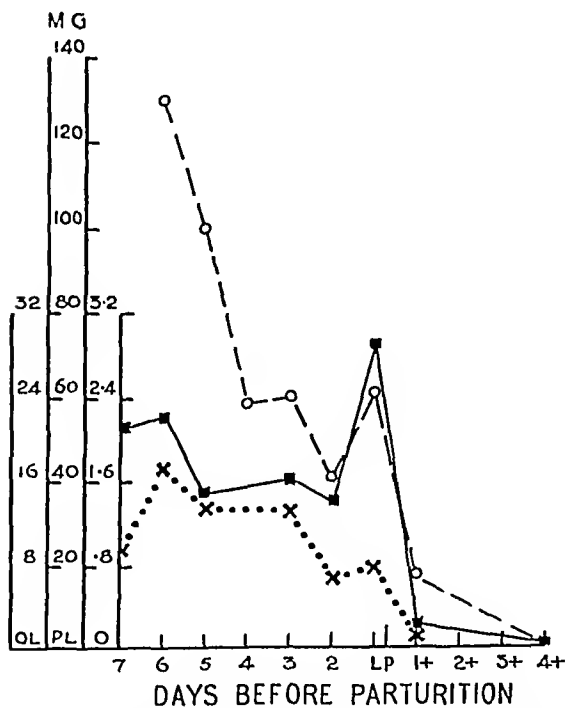


FIG. 9. (Case 65, Table IV) X...X = combined oestriol (OL), ■—■ = combined oestrone (O) and O---O = pregnanediol (PL), L = labour and P = parturition.

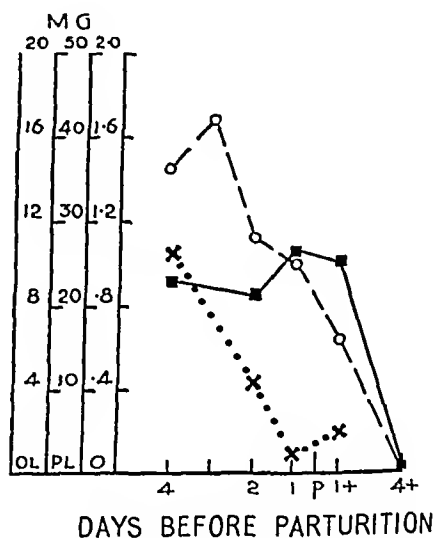


FIG. 10. (Case 64, Table IV) X...X = combined oestriol (OL), ■—■ = combined oestrone (O) and O---O = pregnanediol (PL), P = parturition.

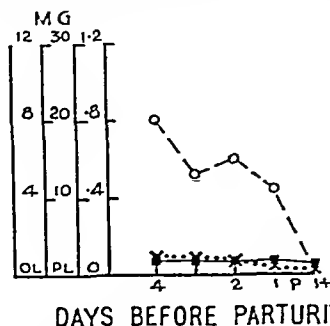


FIG. 11. (Case 66, Table VI. Intra-uterine death) X...X = combined oestriol (OL), ■—■ = combined oestrone (O) and O---O = pregnanediol (PL), P = parturition.

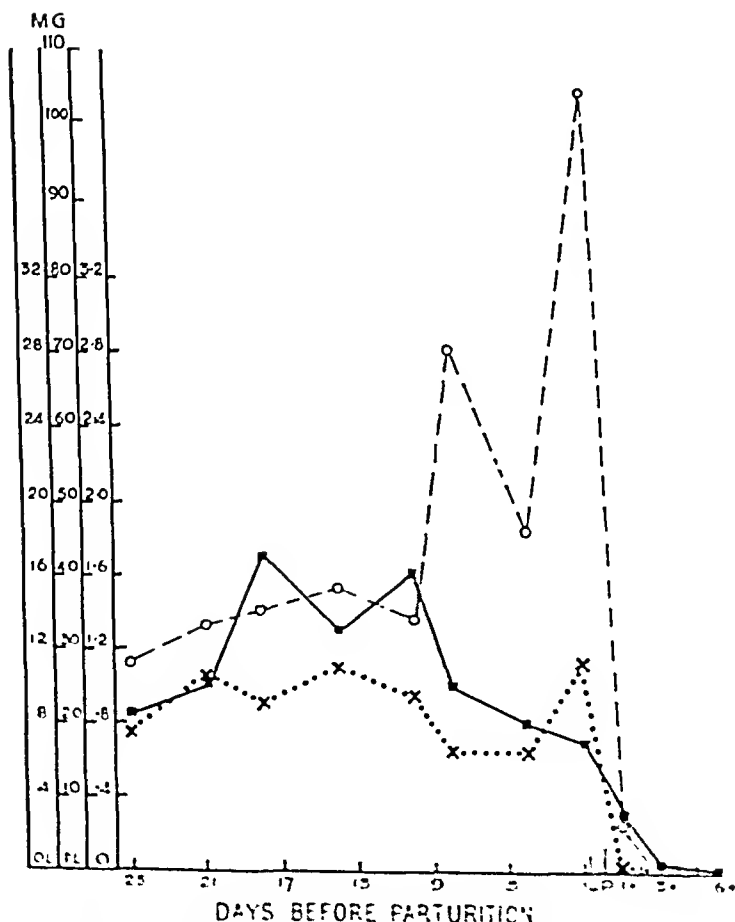


FIG. 12. (Case 62, Table VI. Intrauterine death) X...X = combined oestriol (OL), ■—■ = combined oestrone (O) and O---O = pregnanediol (PL), L = labour and P = parturition.

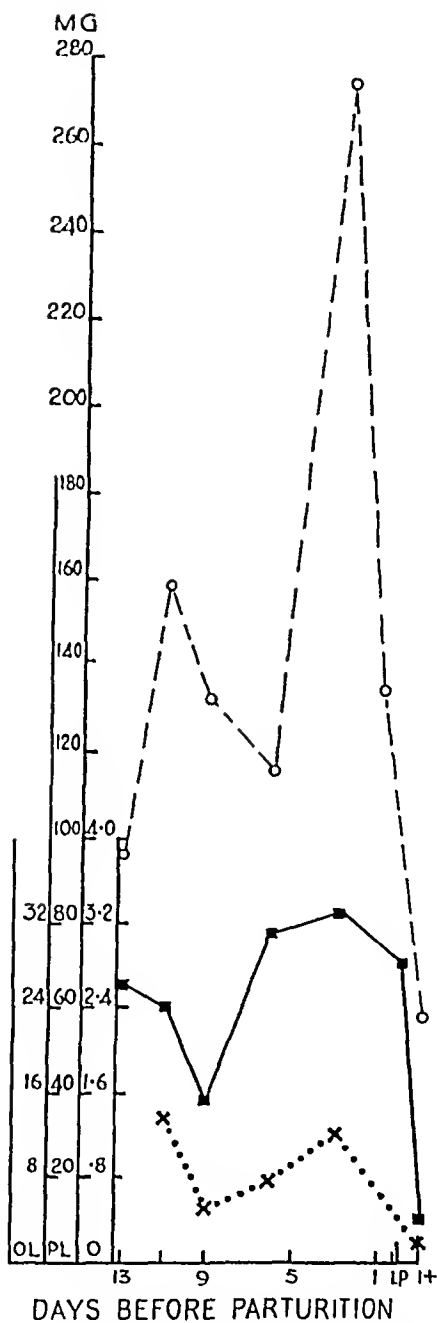


FIG. 13. (Caso 67, Table VI. Severe eclampsia) X...X = combined oestriol (OL), ■—■ = combined oestrone (O) and O---O = pregnanediol (PL), L = labour and P = parturition.

A QUANTITATIVE AND QUALITATIVE TEST FOR STEROID HORMONES BASED ON THE OVIPOSITOR REACTION OF THE FEMALE BITTERLING (*RHODEUS AMARUS* BLOCH)

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UNDER natural conditions, the female bitterling (*Rhodeus amarus* Bloch) has a considerably lengthened ovipositor only during the spawning season (Plate I, Fig. 1). At other times the oviduct is either very short or invisible. Fleischmann & Kann [1932] showed that the ovipositor may be lengthened during the quiescent period by injection of follicular hormone, while Ehrhardt & Kühn [1933] demonstrated that ovipositor growth could be provoked by the addition of the hormone to the aquarium water. Apparently the active principle can be absorbed percutaneously so that injection is unnecessary.

None of the previous investigators has, however, analysed the quantitative relations of the ovipositor reaction so that it may be used for endocrine assay purposes. Such an analysis is undertaken below and the results discussed. These results raise a number of important issues which will be the subject of later publications.

FACTORS INFLUENCING THE OVIPOSITOR REACTION

Expression of ovipositor growth in units

Before considering the actual analysis of the factors influencing the reaction it is necessary to evolve a uniform method for the expression of results.

By observation of the sensitivity of bitterlings having ovipositors of different lengths it was apparent that the lengthening is approximately proportional to the length of the fish. An expression of results based on the actual length of the ovipositor is therefore cumbersome in that a simultaneous record has to be taken of the length of the fish. A convenient method for expressing the length as a proportion of the body-length is to record the length of the ovipositor in terms of the length of the first anal finray. This latter bears a constant relation to the length of the fish and is also convenient, since it can be mentally divided into

eight equal parts and the relative length of the ovipositor quickly and accurately estimated by the naked eye without disturbing the fish. The ovipositor length is expressed in anal fin units (A.U.), each unit being the length of an eighth part of the anal finray. This system of units is used throughout this paper. The relative positions of the ovipositor and finray, and the expression of the ovipositor length in units are shown diagrammatically in Fig. 2.

Accuracy of the observations

Fifty-nine fish had their ovipositors measured 9 times consecutively during 2 hours. To obtain lengths greater than the normal a slow-acting substance was added producing lengths of 5-6 A.U., which do not recede quickly as do those obtained with the usual hormones (this substance was from a particular urine and contains a special hormone or derivative called luteidine [Duyvené de Wit, 1937]).

In 27 fish the recorded ovipositor length was the same in each of the 9 readings. In the other fish there was only one instance of a variation greater than 1 A.U. between the readings. Divergences of 1 A.U. are to be expected if the actual length is midway between two units. It follows that the experimental error in the measurements need not be more than 0.5 A.U. By taking two or more measurements of the ovipositor within a short period the error may be further reduced.

In order to see whether there is any systematic error in the estimations the results of the above observations were plotted as a frequency curve (Fig. 3). Examination of this curve shows there may be a slight preference for the uneven numbers. That this apparent preference is fortuitous is shown in the frequency curve given in Fig. 5. This curve was derived from a similar control series of measurements comprising 749 observations.

Ovipositor growth in relation to time

In order to determine the necessary duration of an experiment to obtain an optimal result an experiment was carried out in which the average ovipositor growth of a number of fish was recorded for several hours under equal, constant, external conditions. The results are recorded in Fig. 4. This shows that for the $5\frac{1}{2}$ hours following the addition of the urine to the aquarium water there was no ovipositor growth, but that during the succeeding hours there was a regular growth of the ovipositor resulting in a straight-line graph. The preliminary $5\frac{1}{2}$ quiescent hours may be termed the latent period; it being understood that the term only refers to the latency of the effect and does not mean that the hormone



FIGS. 1a and b, Female bitterlings during the spawning season with elongated ovipositors.

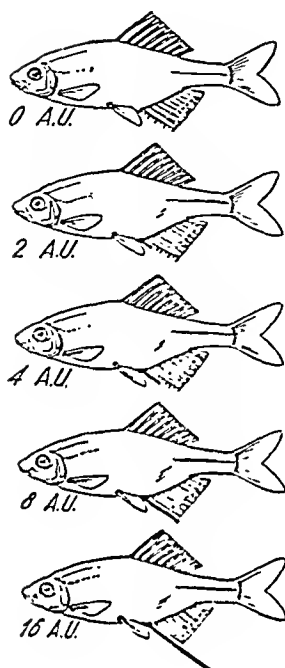


FIG. 2. Diagrammatic representation of the female bitterling with anal finray divided into eight parts showing the length of the ovipositor in terms of anal fin units (A.U.).

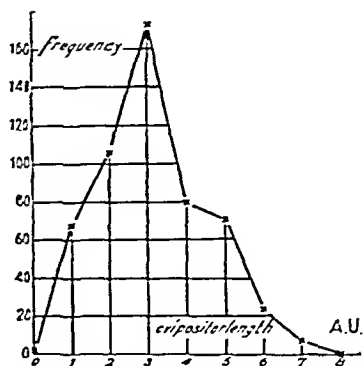


FIG. 3. Frequency curve of 9 consecutive control measurements of ovipositor length in 59 fish.

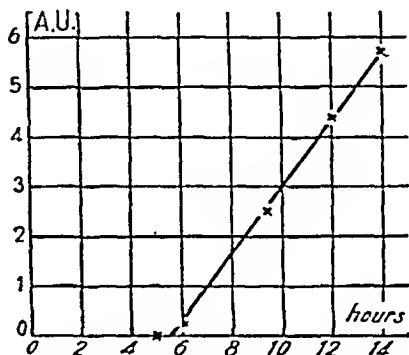


FIG. 4. Average ovipositor growth following the administration of urine. The linear relation between growth and time is apparent.

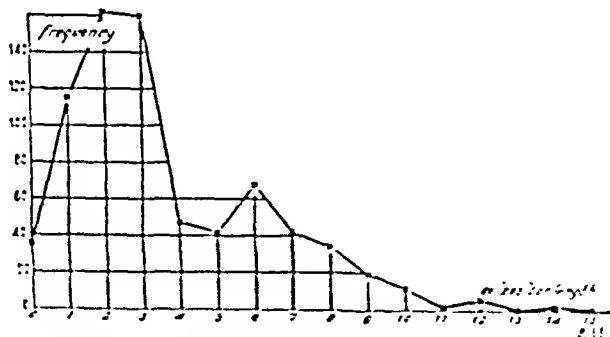


FIG. 5. Frequency curve of a series of 749 control measurements of ovipositor length.

responsible for the effect is not active during this period. Individual records do not show such a straight-line relationship such as is shown in Fig. 4. In Fig. 7 are recorded some of the individual results which when combined formed the average result given in Fig. 4. It follows that to attain an accurate result the mean of a number of individual results has to be determined.

To discover the optimal length of an experiment for assay purposes a further experiment was carried out (Fig. 6) in which a smaller quantity of the same urine as used in the experiment above was added to the aquarium water. It may be seen that there is the same latent period of $5\frac{1}{2}$ hours, a maximum response at 12–14 hours and that beyond this

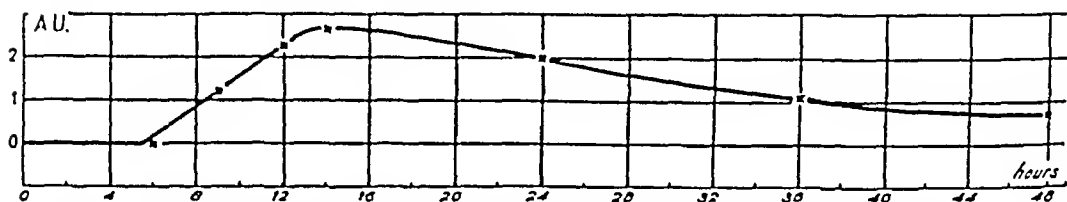


FIG. 6. Growth curve of ovipositor for 48 hours following the addition of urine to the aquarium water in lower concentration than in Figs. 4 and 7.

period there is a progressive fall in the degree of ovipositor length. This experiment demonstrates that the latent period is independent of the quantity of urine added and that to obtain an optimal result the ovipositor length should be measured 12 hours after the addition of the test substance.

Effect of temperature on ovipositor growth

In Fig. 8 are recorded the ovipositor lengths following the addition of the same urine sample to fish kept at different temperatures. Fig. 9 shows the effect of temperature on the ovipositor length measured at different times after the addition of the urine. From the two figures it is apparent that the optimal temperature is 21–22° C., and further that although a higher rate of growth may be obtained during the first 10 hours at 23° C. this temperature has an unfavourable effect in observations taken over longer periods. It will be noted that over the greater part of the range there is a linear relation between ovipositor growth and temperature.

Fig. 8 also shows that there is a progressive prolongation of the latent period as the temperature of the experiment falls. This relation is shown in Fig. 10. The range between 15° and 19° is too short to permit any calculation of the temperature coefficient.

It may be concluded that the optimal conditions for assay purposes are those of an experimental period of 12 hours at 22° C.

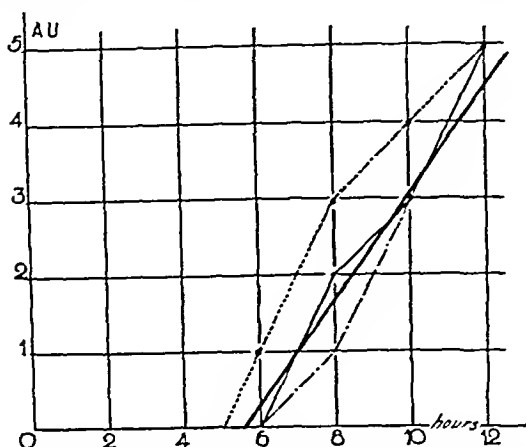


FIG. 7. Some of the individual growth curves obtained during the construction of the mean curve (—).

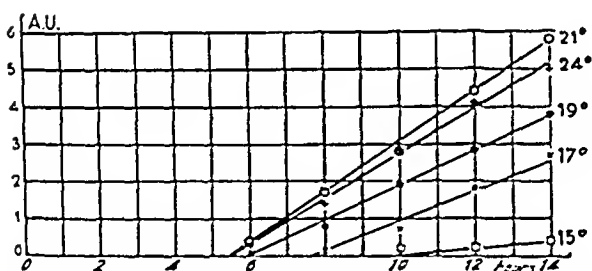


FIG. 8. Growth curve of the ovipositor at different temperatures following the addition of urine.

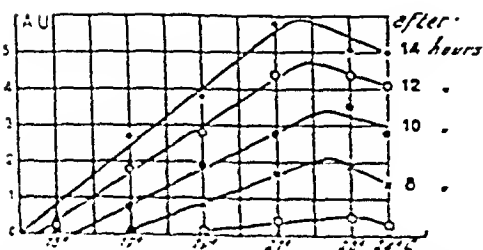


FIG. 9. Ovipositor growth produced by addition of the same urine at different temperatures 6, 8, 10, 12 and 14 hours after the administration.

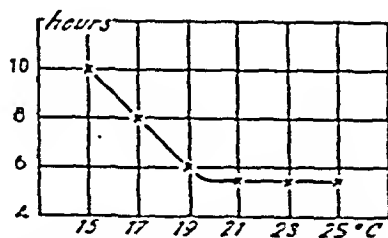


FIG. 10. Relation between temperature of the experiment and latent period.

Relation between ovipositor growth and concentration of test substance

An experiment was performed in which various quantities of the same urine sample were added to the aquarium water. The results are summarized in Figs. 11 and 12. It may be seen that the lengthening of the ovipositor is greater the greater the concentration of the active substance and that between the levels of 0–2.5 ml. of urine per 750 ml. of water the relationship between concentration and response is linear. This shows that within the limits of this dose range the ovipositor reaction is a suitable test for assay purposes. In the case of the actual substance used in this experiment (luteidine) the experimental range extends between 0 and 5 A.U. It is also shown in the figures given that the latent period of the reaction is unaffected by the concentration.

Necessary rest between successive tests

It has been shown above (p. 144) that the ovipositor may recede in the presence of small amounts of active substance. If, however, the concentration of active substance is higher, the maximum ovipositor length may be maintained for much longer periods. When the aquarium is replenished with fresh water during the ovipositor growth there is an abrupt cessation of growth followed by a regression. In the experiment recorded in Fig. 14 fresh water was substituted 8 hours after the start of the ovipositor growth—that is, about 14 hours after the addition of the active substance. It is apparent that the growth of the ovipositor ceased, its length remaining constant for 6 hours and then there was a gradual regression of length, for the succeeding 20 hours, with a final stabilization at 2 A.U. It will be shown below (p. 150) that under these conditions the fish is suitable for another test.

It should further be emphasized that it is necessary to produce ovipositor reactions in the fish at intervals of 1–2 days. If the fish are not so treated, the ovipositor length falls to the quiescent state (0 A.U.), and its capacity to react may be entirely lost or may only be restored by repeated applications of active substance and when the initial length of the ovipositor is again 2 A.U. (see p. 150 below).

Relation between initial length of the ovipositor and sensitivity

A series of 12 fish were tested with 4 successive doses of 3 ml. of urine A. The results were divided into groups according to the initial length of the ovipositor and the average lengthening of the ovipositors in each group calculated. The results are given in Fig. 13, which shows that the response was greatest when the initial length was 1 A.U. and that there was a progressive diminution in the growth as the initial length increased. It

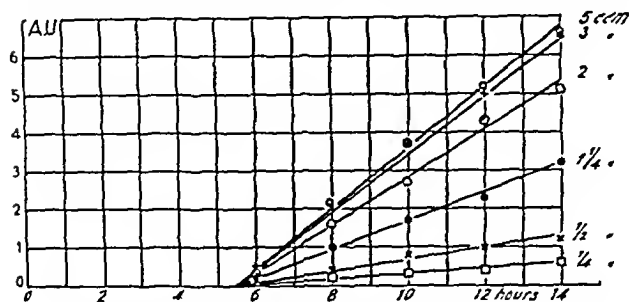


FIG. 11. Effect of concentration of active substance in the aquarium water on the growth of the ovipositor.

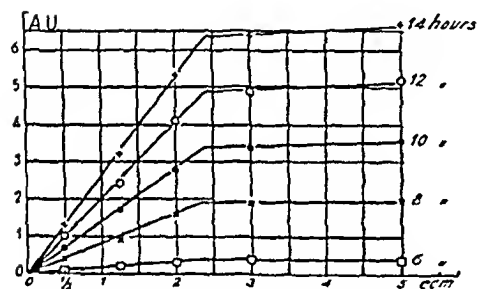


FIG. 12. Ovipositor growth produced by the addition of different amounts of active substance in the 6, 8, 10, 12 and 14 hours following the administration.

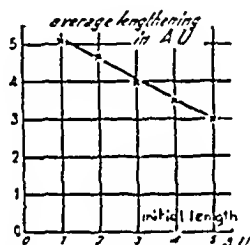


FIG. 13. Effect of initial length of the ovipositor on the response to the same stimulus.

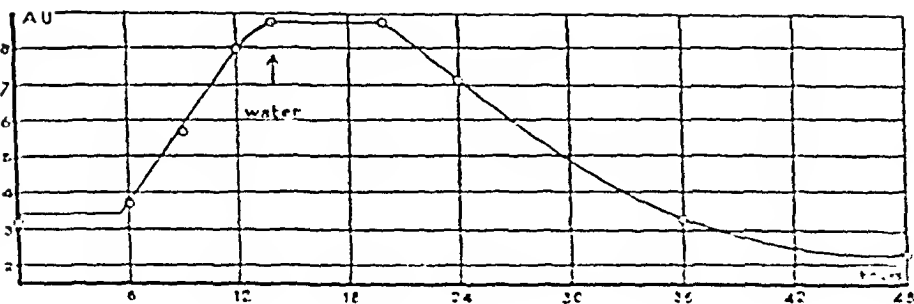


FIG. 14. Effect of replacing the test solution with fresh water.

was also found that this relation was linear, but this finding was not so evident in two other similar experiments.

In practice I use fish with initial ovipositor lengths of 1–3 A.U. and so arrange the group that the average initial length is 2 A.U.

Number of successive tests that may be performed on the fish

A group of 12 fish were given a series of tests at 2-day intervals. The results are given in Table I. It will be seen that the first 4 tests were identical in that 3 ml. of urine A were added to the 750 ml. of water in

Table I. *Effect of successive additions of urine on the ovipositor length of a group of 12 female bitterlings*

Day	Urine given	Vol. of urine (ml.)	Mean ovipositor growth (A.U.)
1	A	3	2.7
3	A	3	4.5
5	A	3	4.2
7	A	3	4.7
9	B	3	0.0
11	A	3	4.3
13	A	2	2.7
15	A	1	1.4

the aquarium. The responses of the ovipositors were, apart from a low response in the first test, practically constant (range 4.2–4.7 A.U.). The cause of the initial low response may be attributed to insufficient sensitization which will be discussed later. Two days after the 4th test 3 ml. of an inactive urine (B) were given and no response was elicited. After this test 3 tests were performed with the original urine A in diminishing dosage. The first test was on 3 ml. and gave a response within the range of the previously determined values. Since it has already been shown that the relation between dose and response is linear within the test range, the results which should be obtained with the doses of 2 and 1 ml. can be calculated. On the basis of the four determinations of the response to 3 ml. (average ovipositor growth = 4.4 A.U.) the growth to be expected with doses of 2 and 1 ml. would be 2.7 A.U. ($= \frac{2}{3} \times 4.4$) and 1.5 A.U. ($= \frac{1}{3} \times 4.4$). In fact, the actual results obtained were 2.7 A.U. and 1.4 A.U. respectively. This means that even after 8 successive tests at 2-day intervals the fish still respond normally.

While it may be true that the sensitivity of the group remains constant during these 8 tests it is possible that this is due to the balancing of rises and falls in the sensitivity of the individual fish comprising the group. To test this point the standard deviation (σ_1) of the results obtained with each fish in the

four tests 2, 3, 4 and 6 were separately calculated. The mean of the 12 standard deviations was 1.19. Similarly, the standard deviations (σ_x) of the responses of four different fish to the same test (i.e. the responses of fish 1-4 in test 2, of fish 5-8 in test 2, &c.) were calculated. The 12 standard deviations thus obtained gave a mean of 1.22. Since the values were nearly the same in the two cases it may be concluded that there was no traceable individual change in sensitivity. The formula used for the calculation of the standard deviation was $\sigma = \sqrt{\left(\frac{\sum(x-\bar{x})^2}{n-1}\right)}$, and though the value of n ($= 4$) was low this is not very important since it was the same in the two cases and the calculations were only used for comparative purposes.

The standard deviations in the above calculations are large compared with the mean values obtained and may cast doubt on the linear relation between dose and response. To settle this point the standard error of the mean was calculated for the tests with 1, 2 and 3 ml. of urine (S.E. of mean $= \sigma/\sqrt{n}$). The results are given in Table II and the graph Fig. 15. The results show that the relation is linear.

Summary: Basis of the assay method

From the above experiments the following points may be indicated as necessary for the performance of a test.

A given quantity of the substance to be tested is added to a given quantity of aquarium water (750 ml. in practice) containing 2-3 fish. The lengthening of the ovipositor is measured in terms of the length of

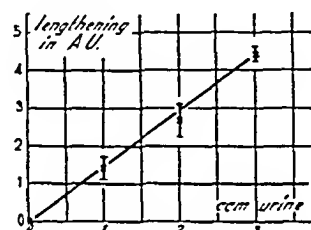


FIG. 15. Dose/response curve of the results given in Table II, including the standard errors of the means.

Table II. Dose/response curve for urine A based on the figures given in Table I

Dose (ml.)	Average ovipositor growth (A.U.)	S.E. of mean (A.U.)
1	1.40	0.29
2	2.67	0.40
3	4.42	0.18

the anal finray (A.U.). The temperature must be maintained constant at 22° C. The ovipositors are measured every 1-2 hours until all growth ceases; then the fish are returned to fresh water. The initial length of the ovipositors must be about 2 A.U. and the fish must have given a positive reaction 1-2 days previous to the test. The quantity of substance to be added must be within the range of the linear part of the dose/response curve. The accuracy of the test varies according to the number of fish used.

PREPARATION OF THE FISH FOR THE TEST

It has been pointed out above that the fish lose sensitivity if left in the quiescent state and it is therefore necessary to increase their sensitivity before an initial test may be made. This may be done by the following treatments.

Growth of the ovipositor on transference to room temperature

When 54 fish were removed from a pond at 4° C. and transferred to an aquarium in which the temperature was raised to 22° within 4 hours there was a spontaneous ovipositor growth, the average ovipositor lengths being 0.4 and 0.8 A.U. 9 and 19 hours after the transference respectively. This finding is illustrated in Fig. 16 which shows that if the growth continues at the same rate an ovipositor length of 2 A.U. would be attained after 2 days. Spontaneous growth, however, never occurs in fish that have reacted to an active substance some days previously.

Shortening of an excessive initial ovipositor length

If the fish are obtained during the months April–July their ovipositors are as a rule long and unfit for assay purposes. If, however, a series of preliminary responses are elicited by the successive application of an active substance it is found that the length of the ovipositors gradually recedes following each application. This fact is shown diagrammatically in Fig. 17. When the initial length has receded to 1–3 A.U. the fish may be used for quantitative assays. It should, however, be pointed out that during the months May–September the fish are less suitable for quantitative work than during the rest of the year.

Sensitization in case of low initial ovipositor length

Fish caught between August and November generally have ovipositors shorter than 1 A.U. and are also unsuitable for quantitative assay purposes. By a similar process of successive stimulations as used in the case of the fish with too long ovipositors the sensitivity can be raised and an initial length of 1–3 A.U. attained. The fish are then suitable for quantitative work. The process is diagrammatically illustrated in Fig. 18.

Sensitization in cases of normal initial length

Fish caught in the months November–March show an ovipositor length of 1–3 A.U., but it is found that in spite of this the preliminary responses to stimulation are generally larger than usual and the ovipositor retains its maximum development abnormally long. It appears that the fish are brought prematurely to the condition found in the spawning season by the application of active substance. A successive treatment with active

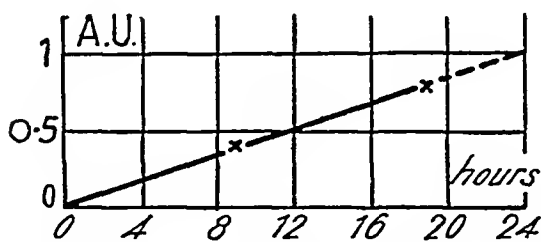


FIG. 16. Growth of the ovipositor following transference to room temperature.

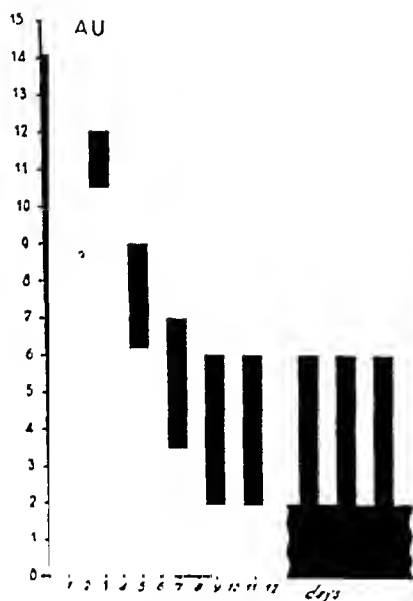


FIG. 17. Diagrammatic representation of the response of fish with elongated ovipositors to a succession of similar stimuli.



FIG. 18. Diagrammatic representation of the response of fish with quiescent ovipositors to a succession of similar stimuli.

substance as in the cases of high and low initial length will bring the fish into a fit state for quantitative work.

Sensitivity level

The above facts demonstrate that whatever the condition of the fish when caught, by a preliminary series of applications of equal quantities of the same urine, the initial length of the ovipositor may be brought to 1-3 A.U. and the sensitivity brought to a condition suitable for the performance of quantitative assays, and further that once this constant sensitivity is attained it can be maintained for some time. If, however, the fish are submitted to a series of weak stimuli the sensitivity tends to decrease but can generally be restored by a further series of adequate stimuli. If the test stimuli are strong or moderately strong, the sensitivity remains constant, but constancy of the sensitivity is only temporal in character and must be checked periodically by the application of some standard preparation.

While on the question of the sensitivity level it is interesting to reconsider the results given in Table I, where the lengthening following the application of 4 successive doses of 3 ml. of urine A in a group of 12 fish was recorded. The average ovipositor growths obtained in the four experiments were 4.50, 4.17, 4.67 and 4.33 A.U. The standard deviations of the results in the four experiments were 0.9, 1.3, 1.9 and 0.8 A.U., and the standard errors of the means were 0.18, 0.25, 0.36 and 0.15 A.U. respectively. If the whole series of 48 results are considered as one group the average growth is 4.42 A.U. It will be seen that the averages 4.50 ± 0.18 , 4.17 ± 0.25 , 4.67 ± 0.36 and 4.33 ± 0.15 A.U. of the constituent experiments all agree with the average of the whole series which is further evidence that the sensitivity remained unchanged.

Unsuitable fish

During July and the early parts of August the ovipositor length is reduced to 0 A.U. in most bitterlings in their natural habitat. Under these conditions even maximal stimuli have hardly any effect on the ovipositor.

Yearly variation in sensitivity

There has been observed a conspicuous difference in the suitability of the fish for test purposes in different years. This fact may be due to alimentary conditions or other factors and is at present under investigation.

APPARATUS AND PROCEDURE

Apparatus

The fish are kept in 36 small aquaria of 750 ml. capacity. The aquaria are placed along the sides of 3 large aquaria which serve as thermostats,

having an electric heating installation. Two or three fish are kept in each of the small aquaria and they may be distinguished from each other if they are of different size. The aquaria have inlet tubes through which air is bubbled, the air being supplied from a pump which is fitted with an alarm setting the pump in action when the atmospheric pressure falls below a certain limit. The whole apparatus (illustrated in Fig. 19)

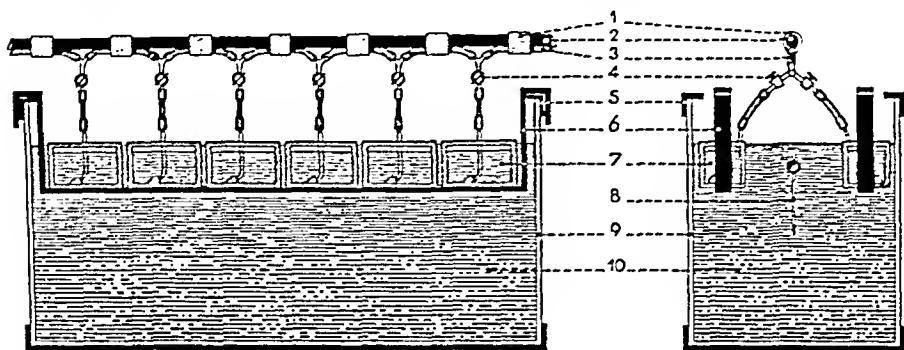


FIG. 19. Front- and side-views of the test-installation. 1. Isolation band; 2. Metal bar; 3. Rubber tube for air supply; 4. Glass conducting tubes with tap; 5. Corner-iron of the aquarium; 6. Metal band on which are fixed small aquaria (7) for test-animals; 8. Thermometer; 9. Glass wall of the aquarium; 10. Water.

is placed in a moderately lighted room with facilities for stronger lighting during measurements.

Preservation of the fish

When fresh fish are placed in the aquaria care is taken to raise the temperature to 22° C. gradually. A rapid rise in temperature is not well tolerated.

The fish are fed dry daphnia, and the water in the small aquaria is replenished twice daily.

Performance of the test

The test is routinely started at 9 a.m., the fish having been previously sorted out and their initial ovipositor length measured. The ovipositors are measured every hour or two hours following the addition of the test substance to the aquaria. As soon as the growth has stopped the fish are transferred to fresh water and the ovipositors measured for the last time. The average growth of the ovipositors in each series is determined and a growth curve constructed.

In cases where toxic preparations are added the fish display apathy or

restlessness, and the experiment is discontinued and lower doses are tried.

Definition of hormone unit

As a unit of hormone action I have taken that quantity of the hormone which, added to 750 ml. of water at 22° C., causes an ovipositor lengthening of 1 A.U. within a certain number of hours, provided always that the sensitivity of the fish has been adjusted as described above. The choice of 750 ml. has been fortuitous owing to the fact that the aquaria used happened to have this capacity. The duration of experiment required to attain the maximum ovipositor length may vary with different active

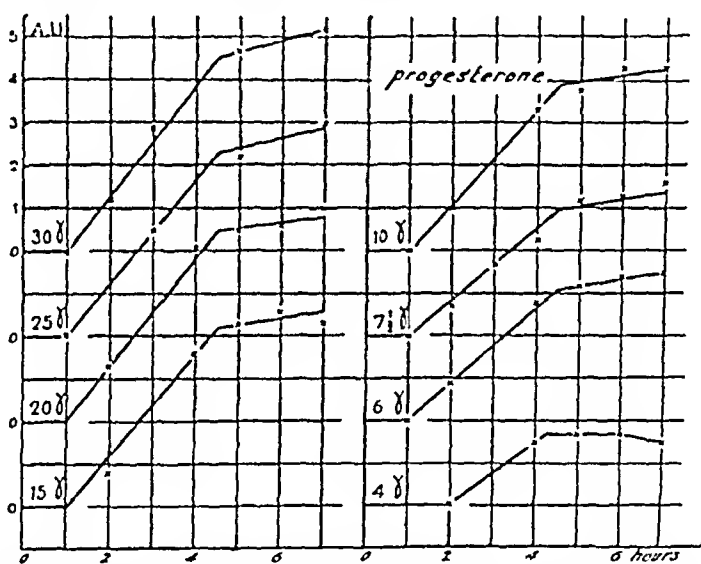


FIG. 20. Response of the bitterling ovipositor to various doses of progesterone.

substances, so that the number of hours in the definition will vary according to the hormone used; I therefore propose to use the following definition for a unit of hormone action: 1 bitterling unit (1 B.U.) is that quantity of an active substance which causes, in n hours, an average lengthening of the ovipositor of 1 A.U. when added to 1 litre of aquarium water.

TEST ON PROGESTERONE

To give an example of the use of the test in practice the following experiment on progesterone may be cited. Doses of 6, 7.5, 10, 15, 20, 25 and 30 µg. of progesterone, dissolved in propylene glycol, were added to the aquaria each of which contained 2 fish. The results represent the averages of the measurements of the response of about 60 fish in each case. The growth curves obtained are shown in Fig. 20. It will be observed

that the latent period with progesterone is only 1 hour compared with the latent period of 5.5 hours in the case of luteidine. The growth ceases in all concentrations 4.5 hours after the addition of the progesterone. From these figures it is possible to construct a dose/response curve for progesterone (Fig. 21). The main characteristics of the curve are the sharp inflexion of the curve at a dose of $9\ \mu\text{g}$. of progesterone and a lengthening of 3.9 A.U. with only slight growth at higher doses [cf. Duyvené de Wit, 1938, 1939].

In future publications it will be shown that the ovipositor of the bitterling responds to the administration of other steroid hormones such

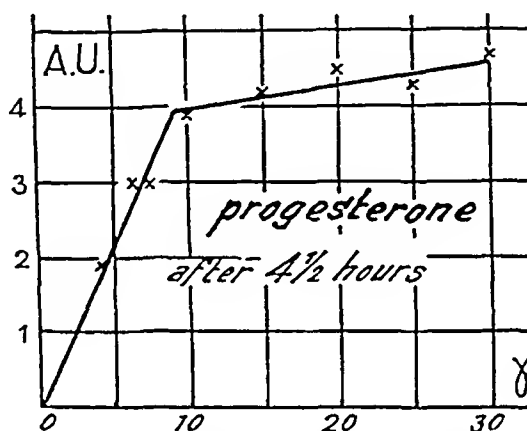


FIG. 21. Dose/response curve for progesterone.

as androgens, oestrogens and adrenal-cortical hormones, and that the growth curves obtained with these hormones have different characteristics. These results have an interesting bearing on endocrinology in general and on the endocrine organization of sex in cold-blooded animals, particularly in the female bitterling.

SUMMARY

A detailed description is given of a quantitative test for steroid hormones based on the response of the ovipositor of the female bitterling (*Rhodeus amarus*, Bloch).

My thanks are due to the 'Jan Dekker Stichting' for grants which enabled these researches to be carried out and also to Messrs. Ciba (Basel) and Organon (Oss) for the supplies of progesterone.

Editor's Note: At the author's request the English translation of this paper was revised by P. C. Williams. During this process certain altera-

tions were made in the drafting, and owing to the present conditions it has not been possible to submit the final manuscript for the author's approval.

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THE EFFECTS OF EXTRACTS OF HUMAN ANTERIOR PITUITARY GLANDS ON *XENOPUS LAEVIS*

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SHAPIRO [1936a] showed that extracts of sheep anterior pituitary glands are gonadotrophic in *Xenopus*. In the female this is seen by the induction of ovulation and oviposition, with induction of hyperaemia of the ovaries, the oviducts and the cloacal labia. Injection of both the male and the female results in amplexus with the production of fertilized ova, indicating a gonadotrophic action in the male as well. Hogben [1930] reported ovulation induced in *Xenopus* by bovine anterior pituitary extracts. This observation was confirmed by Berk & Shapiro [1939], who showed in addition that implanted *Xenopus* anterior lobes were also gonadotrophic in *Xenopus* females, whereas elasmobranch pituitaries gave negative results. Unpublished experiments by the present author, made with material supplied by Prof. Houssay, show that the anterior lobe of the pituitary gland of the South American toad *Bufo arenarum* is also gonadotrophic in male and in female *Xenopus*.

In view of the findings of Shapiro & Zwarenstein [1933, 1934, 1935] that human pregnancy urine extracts can also induce ovulation in *Xenopus*, the report by Shapiro [1936b] that human pregnancy urine extracts can induce the mating reflex when males as well as females are injected and the experiments of Sutherland & Zwarenstein [1939] showing that human pregnancy blood or plasma can induce ovulation in *Xenopus*, it becomes of considerable interest to learn whether the human anterior pituitary gland, which is known to increase in size and in function during pregnancy, is also gonadotrophic in *Xenopus laevis*.

EXPERIMENTAL METHODS AND RESULTS

Adult human pituitaries from male and female Europeans as well as non-Europeans were removed from cadavers at post-mortem and placed in acetone for transport to the laboratory, where the anterior lobes were dissected free, cut up into small pieces, and stored in fresh acetone at room temperature until required for use. Before an extract was made the glands were dried in a current of air and then placed overnight in a crucible over calcium chloride in an evacuated desiccator. The next morning the glands

were extracted. Each gland was macerated for 20 minutes in 6.0 ml. glass-distilled water to which had been added 2.0 ml. 0.1N NaOH. The extract was neutralized with 2.0 ml. 0.1N HCl and the opaque supernatant fluid was used for injection. This fluid frothed on shaking and gave a positive Binret reaction and a negative Molisch's test. Four pituitaries were extracted with glass-distilled water only, the aqueous extract being found to have the same action in females as the alkali extract.

Injection into normal frogs

2.5 ml. of the extract were injected intralymphatically into each of 20 normal female frogs recently brought in from the ponds. Such animals do not ovulate spontaneously under laboratory conditions of captivity, and are therefore suitable for tests of gonadotrophic activity. The dose given was equivalent to one-quarter of one human anterior lobe, and as the volume in which it was contained was considerable, half the dose was injected into the dorsal and the other half into the ventral lymph sac. Each injected animal was then placed in its own glass container half-filled with tap-water and provided with a screw top. The injections were given at 4 p.m. and the result of the experiment was read the next morning. A positive response consisted in finding recently shed ova in the containers. Of the 20 animals injected 15 oviposited and showed hyperaemic activation of the cloacal labia. The remaining five did not oviposit but showed the hyperaemia of the cloacal labia characteristic of the ovipositing female. These five animals were autopsied. Marked hyperaemia of the oviducts and of the ovaries was seen.

Injection into recently hypophysectomized female frogs

Female *Xenopus* were hypophysectomized according to the method of Hogben [1923], the anterior lobe being removed. Two days later injections were made into 10 such animals, the dosage being the same as before. Seven of the 10 animals ovulated and oviposited. The remaining three showed hyperaemia of the cloacal labia, indicating ovarian stimulation. This was borne out by post-mortem examination, when marked hyperaemia of the ovaries and oviducts was seen.

Injection into normal males and females

Males and females were injected with the extract, the females receiving the same dose as before and the males receiving 1.25 ml. of the extract. Fifteen males and 15 females were injected and then arranged in rectangular glass dishes 10" × 8" × 6"; three pairs being placed in each dish. Enough tap-water was added to cover the backs of the animals. The next morning the number of mating animals was counted and some of the shed

ova were removed for microscopic examination. Eleven of the fifteen pairs injected were found in amplexus and large numbers of fertilized eggs from which tadpoles were reared were also found. The matings were of normal type as described by Shapiro [1936a].

Injection into normal males

Eight normal males which had been kept in the laboratory for 2 months were used in this experiment. Before injection the forelimbs were inspected to make sure that no nuptial excrescences ('arm pads') were present. Each frog was then placed in its own glass container and given 3 injections; each injection being given on alternate days. The water in the containers was changed daily and the day after the third injection the arm pads were seen to be fully developed up to the axillae. The animals were then killed and the testes inspected. These were markedly hyperaemic.

DISCUSSION

The active principle in the extract is both water and alkali soluble. The Biuret test result suggests that the particular substance from the human anterior pituitary glands is either protein in nature or else closely associated with proteins. This is of interest in view of the fact that Zwarenstein [1936] and Shapiro [1936c, 1939] have shown that non-protein substances (certain steroids) are gonadotrophic in *Xenopus* females whether normal or hypophysectomized. The active substance in the human pituitary gland does not appear to belong to this group.

With *Xenopus laevis* as the test animal it is not possible at present to distinguish qualitatively between pregnancy urine and pituitary extracts as can be done in the test on immature rats [Hamburger & Pedersen-Bjergaard, 1937].

It is, however, of considerable interest to find that the human anterior pituitary gland is gonadotrophic in *Xenopus* especially in view of the facts that—

- (i) the animal is used in a test for pregnancy, the test depending on the presence of a pituitary-like gonadotrophic substance in the urine [Shapiro & Zwarenstein, 1933, 1934, 1935].
- (ii) the anterior lobe of the pituitary gland is known to increase in size and in function during pregnancy in man and in other mammals [Van Dyke, 1936].
- (iii) human pregnancy blood and plasma have been demonstrated by Sutherland & Zwarenstein [1939] to contain a substance gonadotrophic in female *Xenopus*.

Even if the placenta is to be regarded as the chief source of the gonado-

trophic substances of pregnancy, it would appear that the anterior lobe of the pituitary gland cannot logically be excluded as one of the sources of the gonadotrophic substances found in the pregnant human female.

The injected extract acts by stimulating the amphibian ovary directly and not by activating the animal's own pituitary gland. This is shown by the experiments on recently hypophysectomized animals and by the fact that the action on the secondary sex organs is through the mediation of the gonads as shown by Shapiro & Zwarenstein [1937] in their work on castrates.

Ovulation is associated with the release of amphibian female sex hormone from the ovaries, resulting in hyperaemic activation of the secondary sex organs, the oviducts and the cloacal labia. However, animals which did not oviposit or ovulate showed activation of the secondary sex organs. This suggests that if there is only one active principle in the extract the threshold to induce follicular rupture (i.e. ovulation) is higher than the threshold for ovarian stimulation with the production of oestrogens. The difference in response may be quantitative, smaller doses inducing oestrogenic activity and larger doses inducing ovulation in addition. On the other hand, there is the possibility that there are two active principles present in the extract, one inducing oestrogenic activity and the other follicular rupture. The human extract is follicle-rupturing as well as oestrogenic in the intact female.

The extract is also gonadotrophic in the male. This is shown by the experiments on mating, the development of the arm pads and the hyperaemic activation of the testes. Leslie [1890] and Bles [1901, 1904] have described the nuptial excrescences in *Xenopus*. Berk [1939] has shown that the arm pads do not develop after anterior pituitary injections in the absence of the testes. The effects of the human extract in the male are therefore strictly comparable with the effects in the female, viz. gonadotrophic stimulation of the testes with release of spermatozoa and of androgens in the intact animal.

SUMMARY

- 1 A substance capable of inducing ovulation in normal and in hypophysectomized *Xenopus* has been detected in alkali and in aqueous extracts of human anterior pituitary glands.

2. The extract is oestrogenic in intact females, inducing hyperaemic activation of the oviducts and the cloacal labia.

3. It induces release of spermatozoa in the male with the secretion of amphibian androgens which stimulate development of the male secondary sex characters (the arm pads).

4. The active principle in the extract is either protein in nature or else closely associated with proteins.
5. The possibility that there may be more than one active principle in the extract is discussed.

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THE OESTROGENIC INACTIVITY OF *dl*- α -TOCOPHEROL ACETATE

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Most authors have reported that vitamin E deficiency, at any rate up to quite late stages, has no effect on the ovarian cycle of the adult rat—or presumably of the adult mouse—and it might therefore be anticipated that vitamin E itself would not exhibit the physiological properties of either the follicle-stimulating or the luteinizing hormones. Certain superficial similarities between the action of vitamin E and progesterone, both on experimental animals and in clinical use, make it still more unlikely that vitamin E has oestrogenic activity.

Nevertheless, Underhill [1939] has recently called attention to Verzár's finding [1931] that vitamin E concentrates produce uterine enlargement in immature rats and reported preliminary experiments of his own, in which tocopherol was administered to immature female mice and led to the production of vaginal smears showing oestrus or pro-oestrus. This is in sharp contrast to the still more recent findings of Drummond, Noble & Wright [1939], who injected 5 mg. of *dl*- α -tocopherol acetate (in 25 mg. of olive-oil solution) daily for five days into immature female rats, whose vaginæ were still closed on the subsequent day, and whose ovaries and uteri remained in the infantile condition. Oral administration of vitamin E concentrates to hypophysectomized adult females also failed, in the hands of these authors, to bring about oestrus or pro-oestrus.

As the experiments of Drummond *et al.* were conducted on rats, whereas Underhill used mice, there remains the possibility, however remote, that the different results reported were due to species difference. We therefore think it may be of interest to report our results obtained with mice, after Underhill's statement had been communicated to the Vitamin E Symposium [1939], but before publication of the paper by Drummond *et al.* [1939].

EXPERIMENTAL PROCEDURE

Twenty-six immature female albino mice, from the inbred strain Strong's A2, weighing between 10 and 18 g., were divided into three approximately equal groups; all had closed vaginæ. Into each of the animals in one group 10 mg. of *dl*- α -tocopherol acetate dissolved in 0.5 ml. of olive oil were injected subcutaneously; vaginal smears were taken from these

animals, beginning 24 hours after injection and continuing at 24-hourly intervals for three days or more. A control group of animals received subcutaneously injections of 0.5 ml. of the same olive oil and were examined daily in the same manner. To a third group of animals 0.5 ml. of a solution of *dl*- α -tocopherol acetate (10 mg.) in cod-liver oil was administered orally; the first portion of the dose, 0.2 ml., was fed direct into the animals' mouths, the remainder was mixed in the next day's food. Smears were taken from 24 hours after administration of the second portion.

RESULTS

Table I shows the results obtained. Large numbers of leucocytes were present in every smear, epithelial cells appeared with about the same frequency in all three groups; slight signs of cornification occurred only in one animal in the negative control group. It may be added that 10 mg. of tocopherol (or of its acetate) would seem to be a very large dose for a 20 g. mouse. When added to a basal diet completely devoid of vitamin E, 0.6 mg. of tocopherol acetate will confer on adult rats, weighing 200 to 300 g., a 50% fertility; four times that amount will permit practically all such rats to produce full-term litters [Bacharach, 1939].

Table I

Dose	Route	No. of animals	Nature of vaginal smears						% animals showing oestrus
			After 1 day	After 2 days	After 3 days	After 4 days*	After 5 days*	After 6 days*	
Tocopherol in cod-liver oil	Oral	9	9 L	9 L	9 L	3 L	2 L 1 Le	2 L 1 Le	0
Tocopherol in olive oil	Parenteral	8	8 L	7 L 1 Le	7 L 1 Le	2 L 1 Le	2 L 1 Le	3 L	0
Olive oil	Parenteral	9	9 L	9 L	9 L	2 L 1 CL	3 L	1 L 2 Le	(10)

L = leucocytes only

Le = leucocytes with epithelial cells

CL = cornified cells and leucocytes

* = only three animals in each group examined on the 4th, 5th and 6th days.

In our experience, therefore, a relatively large dose of synthetic tocopherol, whether administered by oral or parenteral route, is entirely without oestrogenic action as tested by capacity to cornify the vaginal epithelium of intact immature female mice.

This is in agreement with the most recent results of Underhill, who reports (private communication) that he has been unable to repeat his former observations. Although those were confirmed by histological examination of preparations made from the experimental animals that had given positive responses, solutions of other samples of α -tocopherol were entirely without oestrogenic effect. Dr. Underhill, who has kindly read the manuscript of this paper, concludes 'I must agree with you that pure α -tocopherol has no oestrogenic action'.

SUMMARY

Synthetic *dl*- α -tocopherol acetate administered orally or parenterally to immature female mice in doses of 10 mg. causes no vaginal cornification.

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THYROID AND BRAIN RESPIRATION

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ROHRER [1924], using the Krogh microrespirometer, found that the feeding of thyroid hormone to mice caused an increase in the O_2 consumption of liver, kidney and muscle tissue. Foster [1926] observed that thyroidectomy produced a diminished O_2 uptake of muscle tissue respiring in glucose, a result also reported by Dye & Maughan [1929*a, b*] with and without the addition of succinate to the respiring muscle mince. Many workers have reported increases in O_2 uptake of surviving tissues after treating animals with thyroxine or thyroid preparations; notably Dresel [1928] in kidney and liver, Anselmino, Eichler & Schlossmann [1929] in liver, spleen and kidney, Hopping [1930] in alligator red blood cells, Hicks [1932] in muscle, McEachern [1932] in heart, Dye [1933] in muscle, Gerard & McIntyre [1933] in liver, auricle and vagus nerve, Meyer, McTiernan & Aub [1933] in liver, Ebina [1932] in liver and kidney, McEachern [1935] in kidney, liver and muscle, Khayyal & Scott [1935] in uterus and Victor & Andersen [1938] in liver and kidney. As far as is known, the only work on the respiration of brain tissue following thyroid treatment is that of Cohen & Gerard [1937]. Using the delayed substrate technique of Quastel & Wheatley [1932], these workers found that when glucose, lactate, succinate or glycogen were tipped into brain brei which had been autolysing for two hours the percentage increase in O_2 uptake was four times as great with thyroid-treated rats as with control animals. This was not so with pyruvate or *p*-phenylenediamine. In the present work increased O_2 uptakes after thyroid treatment were observed in experiments in which the substrate was tipped in at the outset. This was true for glucose, succinate or pyruvate (if ample vitamin B_1 was given) but not for *p*-phenylenediamine.

There are also numerous references in the literature to *in vitro* effects with added thyroxine or thyroid preparations. Vollmer [1923] reported an increased O_2 uptake following the addition of thyroid extract to intestinal mucosa, and Wohlgenuth & Klopstock [1926] described a similar finding for skin. Canzanelli & Rapport [1937] found an increase in O_2 uptake when thyroglobulin was added to liver and, later [Canzanelli, Guild & Rapport, 1939], when added to kidney, testis and heart slices respiring in glucose. Similar effects will be described for brain tissue.

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With thyroxine the evidence for *in vitro* effects is not so clear cut. Positive findings have been reported by Adler & Lipschütz [1922], Neuschloss [1924], Ahlgren [1925], Reinwein & Singer [1928], Ebina [1932], Euler [1932, 1933], Verebely [1932], Paul [1933], Davis, Da Costa & Hastings [1934], Mansfeld [1935], Scott [1935], Davis & Hastings [1936] and Haarmann [1936]. On the other hand, Ellinger [1922], Fleischmann [1927], Anselmino *et al.* [1929], Paasch & Reinwein [1929], Rothschild [1930], Horn [1930], Hopping [1930], Myhrman [1932], Hicks [1932], Verebely [1932], Canzanelli & Rapport [1937] and Canzanelli *et al.* [1939] have reported negative results. Using rat brain tissue the present writer has been unable to observe any significant increase in O_2 uptake following the addition of thyroxine, although the thyroxine concentrations covered a very wide range.

EXPERIMENTAL METHODS

The O_2 uptake was determined by the usual Warburg manometric technique. The animals were killed by decapitation and the tissue prepared in one of two ways. The brain was either (1) minced on a hot plate at $38^\circ C$. with a bone spatula and transferred to a previously weighed bottle and reweighed, as has been the custom in this laboratory, or (2) it was ground in an ice-cold mortar with 4 volumes of cold Ringer-phosphate and pressed through muslin as described by Banga, Ochoa & Peters [1939]. The former preparation is referred to as 'brei' and the latter as 'dispersion'. Duplicate determinations were made in every case.

The reagents were commercial products with the exception of the sodium pyruvate which was made as described by Peters [1938].

The rats used, all males, were taken from the usual laboratory stock, and were fed on the following basal diet:

Rice starch	70
Casein	20
Salt mixture	5
Agar agar	2
Cod-liver oil	3

To 100 g. of this diet were added 10 g. dry yeast.

EXPERIMENTAL RESULTS

O_2 uptake of brain brei from normal and thyroid-treated animals

Rats were given by mouth 0.6 g. desiccated thyroid plus 0.5 mg. crystalline vitamin B_1 hydrochloride per day for 7 to 10 days. Brain brei from animals so treated in the presence of glucose, sodium pyruvate or sodium succinate had a higher O_2 uptake (Table I) than that from controls which received vitamin B_1 alone. If no vitamin B_1 was given the increase was smaller in the case of glucose, and in the case of pyruvate no difference

was observed. This is explained by the fact that thyroid-treated rats on diets low in vitamin B₁ are B₁ deficient, as is evidenced by weight changes following vitamin B₁ administration, catatorulin tests, and cocarboxylase determinations [Peters & Rossiter, 1939].

Table I. O₂ uptake (μ l./g. fresh tissue/hour) of brain brei preparations. Temperature 38° C. The O₂ uptake was measured over period 60–120 min.

Substrate	Treatment	No. animals	O ₂ uptake	Standard deviation	Percentage increase
None	Normal	15	427	± 62	—
	Thyroid	10	447	± 62	5
	Thyroid (No B ₁)	12	450	± 81	5
Glucose 0.01 M	Normal	25	1032	± 199	—
	Thyroid	16	1475	± 161	43
	Thyroid (No B ₁)	12	1275	± 120	24
Sodium pyruvate 0.02 M	Normal	9	1578	± 224	—
	Thyroid	14	1793	± 265	14
	Thyroid (No B ₁)	12	1525	± 229	—3
Sodium succinate 0.1 M	Normal	9	1955	± 200	—
	Thyroid	14	2271	± 265	16
<i>p</i> -phenylenediamine 0.03 M	Normal	5	2200	± 438	—
	Thyroid	3	1666	± 704	—24

With no added substrate there was no significant difference between normal, thyroid-treated and thyroid-treated animals which received no vitamin B₁. With glucose, Fisher's *t* test gave $t = 7.46$ for the difference of means of normal and thyroid-treated animals, and $t = 3.89$ for that of normal and thyroid-treated animals which received no vitamin B₁. In both cases for $P = 0.01$, $t = 2.74$, so that both results are obviously significant. Of less significance, however, is the difference of means of normal and thyroid-treated animals in the case of pyruvate. $t = 1.89$ corresponding to $P = 0.075$, i.e. there is just over 1 chance in 14 of the results being fortuitous. There is no difference between the O₂ uptakes of tissue from normal and thyroid-treated rats which received no vitamin B₁. With sodium succinate, the difference of the means of normal and thyroid-treated animals is again significant ($t = 2.95$). For $P = 0.01$, $t = 2.83$. The few experiments done with *p*-phenylenediamine suggest that there is no increase in O₂ uptake with this substrate following thyroid treatment.

O₂ uptake of brain dispersion from normal and thyroid-treated rats

The animals were treated as in the previous section. Table II indicates that with the dispersion preparation there was no demonstrable increase in O₂ uptake, with or without the addition of glucose.

The in vitro effect of thyroglobulin

Thyroglobulin was prepared essentially by the method of Oswald [1899], but a nucleoprotein fraction was separated by precipitation at pH5 according to the procedure of Heidelberger & Palmer [1933]. The fresh gland was cleaned of as much fat and connective tissue as possible, minced, and extracted twice with 2 volumes 0.9% NaCl. The pH was adjusted to 5 by addition of 0.2 M acetate buffer, and the insoluble nucleoprotein centrifuged off. The supernatant fluid was neutralized with NaOH and half saturated with $(\text{NH}_4)_2\text{SO}_4$. After centrifuging the precipitate was dissolved in 0.9% NaCl. The protein was purified by reprecipitation 3 to 6 times, freed from sulphate by dialysis against distilled water, and dried

Table II. O_2 uptake ($\mu\text{l./g. fresh tissue/hour}$) of brain dispersion. Temperature 38°C . The O_2 uptake was measured over period 60–120 min.

Substrate	Treatment	No. animals	O_2 uptake	Standard deviation
None	Normal	8	253	± 22
	Thyroid	9	239	± 50
Glucose 0.01 M	Normal	8	551	± 89
	Thyroid	9	497	± 48

under reduced pressure. The resulting white powder was soluble in 0.9% NaCl. Three different preparations (A, B and C) were used, and in each case there was an increase in O_2 uptake after adding the thyroglobulin to brei or dispersion preparations (Table III) in the presence of glucose or sodium pyruvate. The effect was very constant, being present in all experiments except the last two with preparation B. For these experiments the thyroglobulin used had been kept in solution for three weeks at 0°C . Several experiments were also done with serum albumen in the same concentrations. This gave no increase in O_2 uptake, so presumably the observed increases with thyroglobulin were not due to a general protein effect. The increase in O_2 uptake with thyroglobulin was only transitory, however, being maximal in the first 30 min., and usually very small by the end of 60 min. (Table IV). This is in direct contrast to the increases after thyroid feeding described above. Here the effect was only slight at first, and was maximal in the period 60–120 min. The effects observed are thus of a different character, and this difference raises the possibility that one of the effects is not that of the physiologically active hormone.

The in vitro effect of thyroxine

Thyroxine was added to both brei and dispersion preparations in the presence of glucose or sodium pyruvate. Many experiments were done covering a wide range of thyroxine concentrations. It is seen that in

two typical experiments (Table V) there is no detectable increase in O_2 uptake following the addition of thyroxine. With saturated thyroxine solutions there was a small decrease.

Table III. *Effect of thyroglobulin added in vitro to brain tissue. O_2 uptake (μ l./g. fresh tissue/hour) measured over period 0-30 min.*

Temperature 38° C.

Substrate	Amount of thyroglobulin	Preparation	No addition	Plus thyroglobulin	Percentage increase
<i>Brei preparations</i>					
Glucose (0.01 M)	10	A	2160	2650	+23
" "	10	A	1630	2120	+30
" "	10	A	2060	2760	+34
" "	2	A	1970	2450	+24
" "	2	A	2190	2270	+ 3.5
" "	2	B	2020	2050	+ 1.5
" "	10	B	2020	2350	+16
" "	2	B	2300	2580	+13
" "	2	B	2130	2110	- 1
" "	2	B	1800	1770	- 1.5
" "	2	C	1720	2060	+20
Sodium pyruvate (0.02 M)	2	A	2930	3150	+ 7.5
" " "	2	A	2790	2940	+ 5.5
" " "	2	B	2180	2330	+ 7
" " "	2	B	2310	2440	+ 5.5
<i>Dispersion preparations</i>					
Glucose (0.01 M)	2	C	1440	1520	+ 5.5
" "	2	C	1210	1400	+16
" "	3	C	1270	1310	+ 3
" "	3	C	1080	1240	+15
" "	3	C	1180	1270	+ 7.5
" "	3	C	1130	1240	+10
" "	3	C	1060	1150	+ 8.5
" "	3	C	1640	1820	+11

DISCUSSION

The increase in O_2 uptake following thyroid administration with glucose or succinate, and the failure to detect an increase with *p*-phenylenediamine agrees with the results of Cohen & Gerard [1937]. The increase with pyruvate, however, is contrary to the findings of these workers. Without the daily administration of large amounts of vitamin B_1 the increase disappeared, suggesting that the failure to obtain an effect with pyruvate may be a result of the vitamin B_1 deficiency associated with experimental hyperthyroidism.

Dye & Waggener [1928] and Sugimoto [1938] found increased cytochrome oxidase (indophenol) contents of heart, liver and kidney tissue of rats, after thyroid treatment. Markoff [1934] observed an increase in liver but not in brain. The fact that there was no increased O_2 uptake

in the presence of *p*-phenylenediamine indicates that this is not the case with brain tissue.

The fact that with glucose as substrate there was a large increase in Table IV. *Comparison between the time relations of the in vivo effect and in vitro effect, produced by adding thyroglobulin, on O₂ uptake (μl./g. fresh tissue/hour). Temperature 38° C. Substrate 0.01 M glucose.*

Treatment	Period (min.)			
	0-15	15-30	30-60	60-120
<i>In vivo effect (average of 25 normal and 16 thyroid-treated animals)</i>				
Normal	2104	1897	1645	1032
Thyroid	2357	2164	1889	1475
Percentage increase	12	14	15	43
<i>In vitro effect of added thyroglobulin (10 mg.). A typical experiment</i>				
Normal	2250	2080	1730	1230
Thyroid	2770	2540	2000	1310
Percentage increase	23	22	15	6.5

O₂ uptake with brei and not with dispersion preparations is interesting. A comparison between the figures of Table I and Table II shows that for the rat, dispersion preparations have only half the respiratory activity of Table V. *The effect of thyroxine added in vitro to rat brain brei. O₂ uptake measured in μl./g. fresh tissue/hour. Temperature 38° C. Thyroxine concentration in g./100 ml.*

Thyroxine concentration	Substrate	
	Glucose (0.01 M)	Sodium pyruvate (0.02 M)
0	1150	1850
Saturated solution	1050	1400
10 ⁻¹¹	1200*	1850*
10 ⁻¹⁵	1100*	1800*
10 ⁻¹⁶	1150*	1700*
10 ⁻¹⁷	1150	1900
10 ⁻¹⁸	1150	1900

* Duplicate determinations not done.

brei. Apparently it is that part of the system which is destroyed by the grinding in a mortar which is influenced by the activity of the thyroid hormone.

SUMMARY

1. In the presence of glucose, sodium pyruvate, or sodium succinate, brain brei from thyroid and vitamin B₁ treated rats has a higher O₂ uptake than brei from controls that received vitamin B₁ only. If no vitamin B₁ is given the increase is smaller in the case of glucose and disappears in the case of pyruvate.

2. With dispersion preparations from similarly treated animals there is no increase in O₂ uptake.

3. *In vitro* addition of thyroglobulin causes an increase in O_2 uptake of brei and dispersion preparations of rat brain with both glucose and sodium pyruvate. This effect is considered distinct from that of 1, because of the dissimilarity in time relations.

4. *In vitro* addition of thyroxine does not cause an increase in O_2 uptake of brei and dispersion preparations of rat brain in the presence of glucose or sodium pyruvate.

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A REPLY TO RECENT CRITICISMS OF THE THEORY OF A RELATIONSHIP BETWEEN VITAMIN E AND THE OESTROGENS

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RECENTLY Drummond, Noble & Wright [1939] and Cuthbertson & Drummond [1939] have criticized the method employed by the author in determining relationships between vitamin E and blood oestrogens. It would seem appropriate to discuss the questions raised in these papers and to present such further evidence as has accrued to the subject.

In the article by Cuthbertson & Drummond a number of *a priori* objections to the validity of the test are presented. It is stated that the reaction produced can scarcely be due to proteolysis by trypsin because there is no initial increase in the formol titration. However, this characteristic of tryptic digestion was observed years ago by Cole and confirmed by both Wigglesworth [1928] and Fine [1931]. Fine held that trypsin had two active components: a tryptase which acted primarily to liberate free acid and a polypeptidase, which subsequently yielded amino acids. Northrop & Kunitz [1932] say: 'The increase of formol titration . . . measures principally the later stages of digestion and . . . can hardly be considered as the result of hydrolysis of the protein itself.' As the preliminary phase of the digestion only is pertinent to the technique of the writer's test, it is conceivable that no amino acids may appear within the period of observation.

Further, Cuthbertson & Drummond express doubt that trypsin could be responsible for any 'digestion' produced, because the author claimed that acid production might occur over a temperature range of 20-90° C.—although, needless to say, these were not suggested as optimum temperatures for the digestion test. It is to be noted that the solutions are exposed to the ferment for only 40 minutes, and even temperatures considerably higher than 65° take at least this time to inactivate trypsin, especially if a crude preparation is used and it is in acid solution, both of which conditions decrease the lability of trypsin. The experiments of Mellanby & Woolley [1913] and Eddie [1914] showed that trypsin in dilute acid solution could be heated to 100° C. with very little loss in activity. The upper temperature ranges mentioned above were used in order to inactivate antitrypsin, an alternative explanation of the phenomenon observed.

The digestion of trypsin-serum-buffer mixtures produces a gradual rise of acid. The digests are then titrated against alkali, using phenolphthalein for indicator. Many observers have difficulty in detecting slight changes in pink colours. Possibly some other indicator would prove more generally useful, although it is hard to visualize a more readily detectable end-point than that afforded by phenolphthalein. Fine said of this indicator under similar conditions: 'The end-point was remarkably sharp.' The writer found the indicator mixture evolved by Cuthbertson & Drummond much less satisfactory. When phenolphthalein is used it may be difficult to detect the actual end-point, but it is easy to ascertain when one has gone one drop *beyond* it. It appears to the writer that the curves published in Cuthbertson & Drummond's paper (Figs. 1, 2, 3) merely indicate difficulty in determining an end-point. I have had many such curves sent to me by those first attempting the test, have then had these workers do the test under my own supervision and thereafter they have secured satisfactory results.

How 'absorption of CO_2 ' could account for such variations in the digests as Cuthbertson & Drummond observed is difficult to understand, since the tubes used in the digestions are small, are more than half-filled with fluid, and are kept corked almost throughout the test. It would be still more difficult to believe that a potent trypsin solution could be in contact with serum protein and produce no evidence of digestion at all after even a 'period of two hours and 20 minutes'.

The author's method is designed to detect small initial variations in digestion rate and is more sensitive than a viscosimetric technique. It does not invalidate the results to say that a viscosimetric method, where a number of additional components were involved, failed to show changes analogous to those seen in the titrations. The titration method was deliberately chosen because of its sensitivity and simplicity.

A qualitative test for blood oestrogens or E-deficiency (assuming for the moment one test can detect both) must be only a temporary phase of the development of this subject. However, one can bring forward such sound objections to any conclusions based upon studies of urinary oestrogens only [Polonsky, 1936; Hain, 1939; Moller-Christensen & Pedersen-Bjergaard, 1936], or upon feasible biological assays [Freed, Hechter & Soskin, 1939; Hechter, Lev & Soskin, 1940; and Fluhmann, 1936], that a simple and rapid chemical test upon blood such as the author has attempted to formulate should not be discarded prematurely. Several writers have reported that they have found the test unsatisfactory; others have used it successfully. The latter have usually been those trained in the technique by its originator.

In the paper by Drummond, Noble & Wright [1939], the authors

discuss the physiological basis of the theory that vitamin E acts in the organism principally as an antagonist of oestrogens [Shute, 1936], or to be more specific, that the evidences of E-deficiency are produced by the liberation of an excess of oestrogens. Mention is made, indeed, of Underhill's [1939] work which indicated that vitamin E actually was oestrogenic. Neither Demole [1939] nor Drummond *et al.* [1939] could, however, confirm Underhill's observations.

That E-deficient rats continue to exhibit fertility and oestrous cycles, as Drummond *et al.* point out, is no argument against the theory that oestrogens are liberated by prolonged E-deficiency. Wade & Doisy [1935] gave large daily doses of theelol to forty-one female rats over periods of as long as 316 days, and consistently observed the early re-establishment and continuation of oestrous cycles and that there was unimpaired fertility during such treatment. They found, too, that uterine and ovarian weights revealed an initial decrease during the course of their experiment, but soon returned to normal.

In 1937 the writer published a note [Shute, 1937a] on one of the interesting early pregnancies which give a negative Friedman test. In this case the administration of vitamin E was accompanied by a rapid change, over a period of only 48 hours, to a strongly positive Friedman. If this change can be fairly ascribed to the vitamin E that had been given, it certainly suggests that it neutralized an oestrogen excess, allowing prolactin to be excreted as in most normal early pregnancies.

However, it would not be surprising if E-deficiency displayed certain specific characters, and from Mason's [1933] work it appears that the effect on the testis is dissimilar to that produced by oestrogen administration. The studies of Einarson & Ringsted [1938], Bicknell [1940] and others suggest, too, that it has a specific action on certain portions of the spinal cord and its nerve roots, as well as on the skeletal musculature. Bicknell points out that this is not due to α -tocopherol but to some other factor in the wheat germ. However, Wechsler [1940] has secured therapeutic results in amyotrophic lateral sclerosis with synthetic *dl*- α -tocopherol. Moreover, the very close analogy between the gynaecological and obstetrical properties of vitamin E and those evinced by progesterone calls for an attempt at a single explanation, the most obvious being that both are effective anti-oestrogens.

The writer has used his proteolysis test upon blood samples taken from nearly 2,000 women showing many different obstetrical and gynaecological conditions, and has consistently found it feasible to consider vitamin E as an anti-oestrogen, in this field at least. It has been possible to time the oestrogen therapy of certain secondary amenorrhoeas and predict the time when menstruation should recur. Weeks, or even months,

before spontaneous abortion or miscarriage or a late toxæmia of the haemorrhagic type [Shute, 1939a] has developed clinically it has been possible to predict that such a complication lay in the offing. Late pregnancy toxæmias have been distinguished on this basis when no other differential evidence could be discerned and appropriate therapy, widely different for the two main types, has dispelled the incipient toxæmia in many instances. Cases of senile vulvovaginitis have been analogously differentiated and treated. Many of the blood specimens studied by us have come from outlying points and from patients we have never seen. We know of no instance in which the advice based upon our test has led to improper therapy, although, for example, senile vulvovaginitis associated with a high blood oestrogen, is, to say the least, not helped by the administration of oestrogens. The same remark is even truer of the late pregnancy toxæmias. The clinical field in which a blood oestrogen test has value is enlarging steadily. It has an essential place in the management of hyperemesis, dysmenorrhœa, menorrhagia and many post-menopausal complaints, and in differentiating acute appendicitis and placenta prævia from premature placental detachment. It may be added, in passing, that the test appears to have no significance in cases of habitual abortion. In ten such personal cases, vitamin E in any dose has been valueless. Yet three of these have since carried pregnancies far beyond the usual time of abortion without the assistance of E! These pregnancies have not yet reached term. We define habitual abortion, of course, as the condition in which at least the last three abortions have occurred consecutively at or before the third month. Because this definition has not been adhered to, much of the literature on this topic is fallacious.

For some years reports have appeared which indicate that post-menopausal women often or occasionally excrete oestrogens in the urine [Shute, 1937b]. The writer has indicated recently [Shute, 1939b], entirely on the evidence derived from his proteolysis test, that at least 67% of 82 post-menopausal women (quite inadequately tested for the presence or absence of cycles) and 75% of 12 of these tested rather more completely, revealed blood oestrogens cyclically or occasionally even long after the cessation of the menses. If more trials had been feasible, it is fair to assume that this percentage would have been appreciably raised. Fluhmann & Murphy [1939] still more recently gave a figure of 89%, derived from a study of 76 such women, using Fluhmann's innocification test (a histological assay technique) and checking the women adequately.

Smith & Smith [1935] were the first to indicate that the blood of eclamptic women revealed low oestrogen values. This observation was confirmed by the writer by means of the proteolysis test, and later by

Mühlbock [1939]. The writer described the beneficial effects of oestrogens in the treatment of these cases [Shute, 1937a] and now has observations on ten true pre-eclampsies and six convulsive eclampsies to confirm his first impression. Siegler [1939] has also used this form of therapy with satisfactory results.

It is an easy inference from the theory of E-oestrogen interbalance that if a true pre-eclamptic woman with low values for blood oestrogen be given vitamin E, her blood oestrogen values should fall still lower; if this oestrogen level has any relation to her pathological state perhaps convulsions should then appear.

One of the writer's patients developed a fulminating toxæmia ending in convulsions when large doses of wheat germ oil had been administered without the precaution of a preliminary test of blood oestrogen level. Unfortunately there is a large gap in the chain of evidence and inference here, but a colleague has had two similar experiences and Barrie [personal communication, 1940] has found that some of her rats have displayed clonic convulsions after the administration of large doses of α -tocopherol; these animals at autopsy showed the histological changes she had earlier reported [Barrie, 1939] as bearing a resemblance to those of toxæmic human pregnancy.

If vitamin E and the body's oestrogens are in modifiable equilibrium throughout the year, summer and winter diets should alter the results of oestrogen assays. Such a seasonal change in the bloods of normal male medical students had been observed by the author [Shute, 1938]. A more or less corresponding observation on castrated mice has been reported by Duszynska [1938], who found that more oestrogen was required to produce changes in these animals during the summer months than in the late winter.

In a series of 63 dysmenorrhœic women, taken at random, the writer found high blood oestrogen values, by the use of his test, in 49% [Shute, 1940]. Kotz & Parker [1937] tabulated their results in 100 such women and reported a figure of 68%. They used the Frank-Goldberger technique.

All of the above evidence indicates that sufficient confirmation of the writer's deductions from both theory and practice has already appeared to stretch the long arm of coincidence very far indeed. It is improbable that these conclusions have been derived from the use of methods involving an excessive margin of error.

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THE ASSAY OF PROLACTIN BY MEANS OF THE PIGEON CROP-GLAND RESPONSE

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Of the various physiological responses to prolactin the most characteristic and unequivocal is its ability to cause thickening of the crop-glands in doves and pigeons [Riddle & Braucher, 1931; Riddle, Bates & Dykshorn, 1933], and this response is now widely used for estimations of potency. Assay methods based on the crop-gland response fall into three main classes, (i) methods involving determination of the weight of the stimulated crop-glands as first proposed by Riddle *et al.* [1933], (ii) systemic minimal stimulation methods such as that of McShan & Turner [1936], and (iii) local minimum stimulation or 'intradermal' methods originated by Lyons & Page [1935]. The chief value of the micromethod of Lyons & Page is that it will detect minute amounts of hormone. For accurate assay, however, we prefer the crop-weight method to methods involving determinations of the minimal stimulation dose since the latter depends on a subjective criterion while crop-weight determinations are, of course, objective.

Riddle and his co-workers have studied many of the factors which affect the crop-gland response [cf. Bates, 1937; Riddle & Bates, 1939] and since important ones are race or breed of bird, age and body-weight, it is clearly preferable to work with birds of standard breed and age. Unfortunately, in England it is at present impossible to obtain adequate supplies of such birds, so that workers in this country, who wish to carry out prolactin assays, must do the best they can with 'mixed' pigeons obtained from dealers. This paper deals with data which have accumulated in the course of numerous prolactin assays, and summarizes our experience with the crop-weight method of assay as carried out under the conditions obtaining in this country.

METHODS

The systemic crop-weight method of assay has been used throughout, the technique of injection, and of dissection and weighing of the crop-glands being similar to that of Rowlands [1937]. Each bird received six daily injections of hormone, the daily dose being dissolved in 1 ml. aqueous medium and injected subcutaneously in the axillary region where the skin

is loose. Since the route of administration affects the response [Bates & Riddle, 1936], care was taken that the solution was always injected subcutaneously and not into the deeper tissues or the skin. If the solutions are more alkaline than pH 9, the damaged tissue thickens round the site of injection, and the solutions were therefore neutralized almost to the point of precipitation of the hormone.

In choosing birds for assays, more than the requisite number of birds were bought and weighed the day before the first injection. The groups were so made up that the body-weight range and mean weight for each group were as nearly equal as possible, and latterly birds heavier than 400 g. or lighter than 250 g. were rejected. Except where otherwise stated the birds were kept in an unheated room and fed *ad lib.* on wheat and water.

The birds were killed and weighed 24 hours after the last injection, food having been withheld overnight in order to ensure empty crops. The crop-glands were dissected out, scraped free from adherent secretion, fixed in Bouin's fluid for 24 hours, transferred to 70% alcohol and weighed after 2-3 hours.

RESULTS

The dose-response curve

When the international standard of 'lactogenic hormone' (i.e. prolactin) was made available it was decided to construct a curve of reference for assay purposes. The data for this curve were obtained from groups of 10 pigeons kept under identical conditions and injected together. The results are plotted in Fig. 1 both for absolute crop-weights and for crop-weights expressed as percentages of body-weight (relative crop-weights). Both curves are sigmoid in shape if the uninjected controls are included. If, however, the responses to the different doses of hormone are plotted against the logarithm of the dose, both sets of points fall on good approximations to straight lines (Fig. 2) the fit being better when the results are expressed as percentages of the body-weight. The equations of these straight lines are $y_1 = 3.143x - 0.201$ and $y_2 = 1.111x - 0.069$ where y_1 is the crop-weight in g., y_2 the crop-weight expressed as a percentage of the body-weight and x is log total dose in I.U. From the approximately rectilinear relation of crop-weight to log dose it may be concluded that the crop-weight method is suitable for assay purposes at least over the range of total dose from 3 to 18 I.U.

It is of interest to compare the slope of our log dose-response curve with that given by Riddle *et al.* [1933] for their assay method involving four daily intramuscular injections with autopsy on the 5th day. The results of these workers were found to conform to the equation $y = 765x + 210$, where y = crop-weight in mg. per 150 g. body-weight and x = log dose of

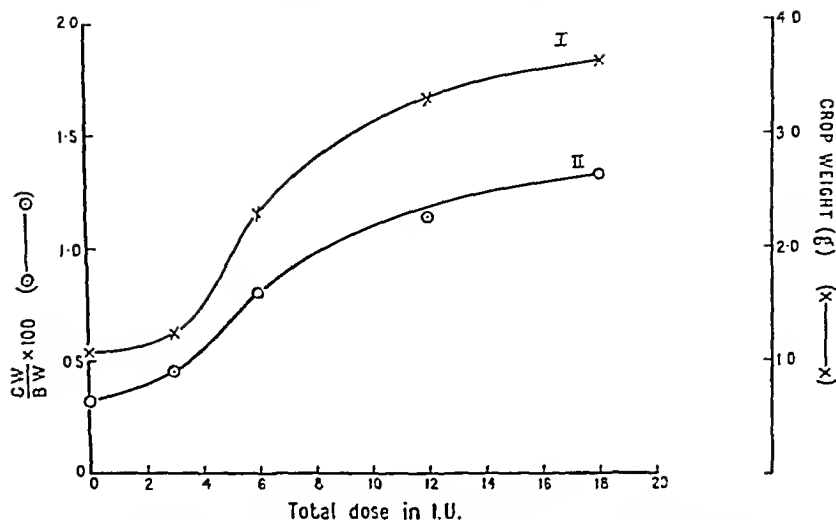


FIG. 1. Response of pigeon crop-gland to prolactin. Curves showing relationship between total dose of prolactin (International Standard) and mean (I) absolute crop-weight (X) or (II) crop-weight expressed as a percentage of body-weight (O).

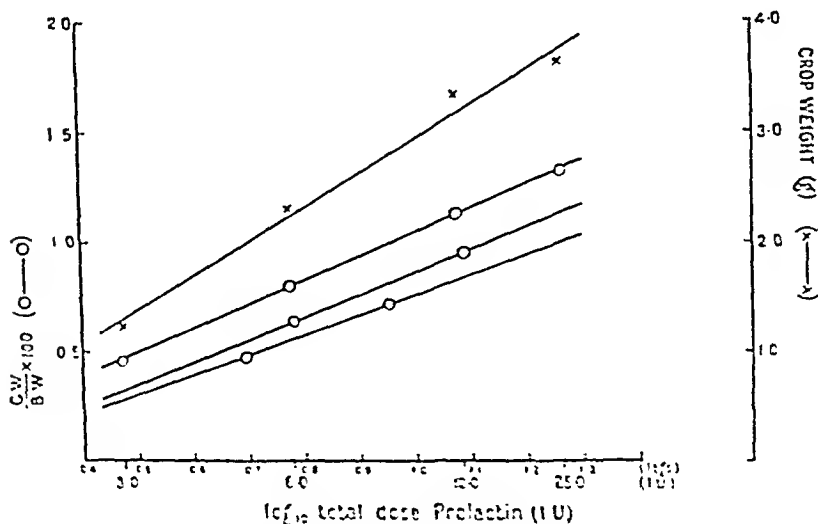


FIG. 2. Response of pigeon crop-gland to prolactin. Relation between mean weight of stimulated crop-gland and the logarithm of the dose of International Standard prolactin.

prolactin in mg. per 150 g. body-weight. Recalculating our straight line to the same basis we have $y = 1685x + 341$, from which it is seen that the slope of our regression line is more than double that of Riddle *et al.* The two sets of results are not strictly comparable since the two experiments were made at different times and in different hemispheres; nevertheless it seems fair to conclude that the six-day method, though more time-consuming than the four-day method, possesses the undoubted advantage of greater sensitivity.

Two of us (F. J. D. and S. J. F.) have independently had occasion to determine the response to two doses of the international standard in the course of subsequent assays which were conducted in our respective laboratories. These results are also plotted in Fig. 2, and it will be seen that in both cases the slope of the line was practically the same as that of the original log dose-response curve though the position of the line had changed. As was to be expected, the mean response of groups of 'mixed' pigeons alters from time to time, and this emphasizes the necessity of making a simultaneous test of the standard of reference every time that a substance is assayed. Since the slope of the dose-response curve does not appear to alter very much from time to time, it is probably quite sufficient to compare the unknown with one dose of the standard only.

The degree of accuracy of the assay

The accuracy of an assay of the type under consideration depends on three factors: the standard deviation of a single observation, the number of birds used in each group and the slope of the dose-response curve. In general, the greater the slope of the curve, the more accurate the method of assay. This statement is subject to the limitation that with a very steep slope the response for practical purposes ceases to be graded and becomes quantal.

From the data obtained with the international standard in the course of the construction of the standard curve, the probable limits of accuracy of the assay have been calculated by the method given in the British Pharmacopoeia Commission, Reports of Committees No. 10, 1936, for the three levels of significance, $P = 0.67$, $P = 0.95$, and $P = 0.99$ and for comparisons between groups containing 5, 10, 20 and 100 birds. These limits, both for absolute and relative crop-weights, are given in Table I, together with similar data, for absolute crop-weights only, calculated from the results of numerous experiments carried out with different doses of a prolactin preparation AP52C on various occasions over 2-3 years. It may be mentioned at this point that in some of the earlier experiments with AP52C, which were primarily carried out in connexion with another investigation [Folley, 1939], the birds received at the same time as the

prolactin injections, subcutaneous injections of 0.5 ml. or 1 ml. sesame oil. There is no reason to suppose that such injections had any effect on the response of the crops to prolactin.

Table I. *Limits of error of an assay of prolactin obtainable by the use of (a) crop-weight expressed as a percentage of the body-weight; (b) crop-weight—all the pigeons being injected with standard or test substance at the same time and (c) mean crop-weight of an aggregate group of 238 pigeons, comprising smaller groups injected at different times*

Criterion	No. of pigeons given		Limits of error (%)* for:		
	(a) Standard	(b) Test subst.	$P = 0.67$	$P = 0.95$	$P = 0.99$
(a)† Crop-wt. $\times 100$	5	5	80-125	63-158	56-180
Body-wt.	10	10	85-118	72-138	66-162
(Tests at the same time).	20	20	89-112	80-125	78-134
	100	100	95-105	90-111	88-114
(b)† Crop-weight. (Tests at the same time.)	5	5	78-129	60-166	52-192
	10	10	84-120	70-143	63-159
	20	20	88-114	78-129	72-139
	100	100	95-106	89-112	86-116
(c)‡ Crop-weight. (Tests at different times.)	5	5	68-148	42-240	36-274
	10	10	76-132	58-174	49-204
	20	20	82-122	68-148	60-166
	100	100	92-109	84-120	80-125

* Limits of error. The method of stating the limits of error is that employed in the Addendum [1936] to the British Pharmacopoeia.

† Criteria (a) and (b) are based upon data from experiments in which groups of 9 or 10 pigeons were injected with the following total doses of International Standard Prolactin Powder: 0.3, 0.6, 1.2 and 1.8 mg. per bird.

‡ Criterion (c) is based upon data from experiments in which groups of birds were injected at different times (spread over a period of 2 years), with prolactin preparation AP52C, in the following total doses (the numbers in brackets being the aggregate of birds on the dose): 6 mg. (18), 13.5 mg. (19), 9 mg. (57), 10.8 mg. (13), 18 mg. (113) and 24 mg. (18).

It is clear from general inspection of the table that the limits of accuracy obtainable with this assay compare favourably with other biological assays even though the only available experimental animals are less uniform than those available for most biological assays. At all levels of significance and for all sizes of groups there is evidently a slight advantage in working with relative crop-weights than with absolute crop-weights. The probable limits of accuracy at the above three levels of significance calculated from the relative crop-weight data obtained with the international standard are plotted in Fig. 3, which nicely illustrates the fact that the increase in accuracy thereby attained makes it well worth while using groups of 15-20 birds rather than of 10, but that the use of groups larger than 20 hardly justifies the expense involved.

Relation between crop-weight and body-weight for a given dose of hormone

As we have shown above, the probable limits of accuracy of prolactin assays are slightly decreased if the calculations are made on the basis of crop-weights expressed as percentages of the body-weight. This indicates the existence of a correlation between crop-weight and body-weight for a given dose of hormone. That this correlation exists has been claimed by

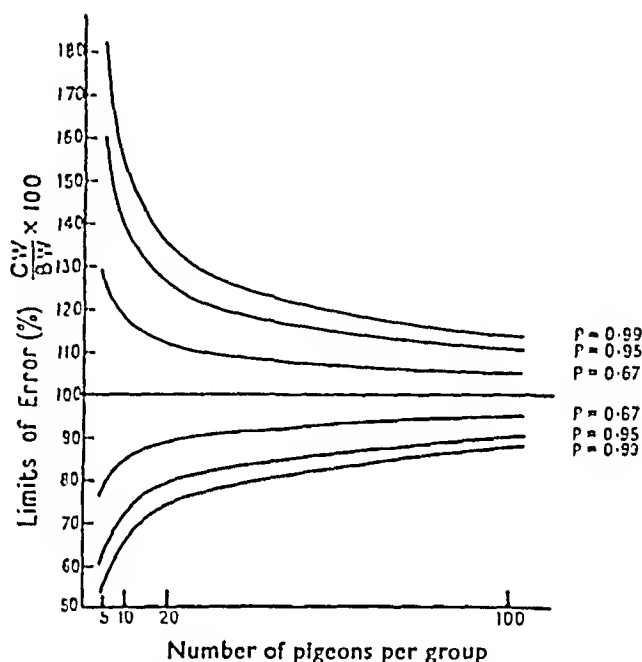


FIG. 3. The accuracy attainable in an assay of an unknown preparation of prolactin by the use of groups of different numbers of pigeons, the two groups being of equal size in each assay and one group being used for the standard and one for the unknown preparation.

Riddle *et al.* [1933] and Rowlands [1937], but, in our view, on rather indirect evidence. We therefore thought it worth while to analyse some of our data from this point of view. In Table II are given the correlation coefficients between crop-weight and body-weight for two doses of preparation AP52C. Each large group is a composite one made up of smaller groups of birds injected at different times with the same dose of hormone.

At each dose level, r has the approximate value 0.5 and is highly significant. The existence of a positive correlation between crop-weight and body-weight for a given dose of hormone, in groups of 'mixed' pigeons can thus be taken as established. This emphasizes the distinct advantage of using relative crop-weights in prolactin assays unless the body-weights of the birds used are kept within rather narrow limits.

Variations in the response to a given dose of hormone

The mean responses of groups of pigeons to two doses of AP52C, determined on various occasions, have been collected in Table III.

Table II. *Correlation between crop-weight and body-weight at post mortem*

Date of 1st injection	No. of pigeons in group	Range of body-weight at post mortem (g.)	<i>r</i>	<i>P</i>
(a) Total dose = 9.0 mg. AP52C (approx. 8 I.U.)				
27/5/38	18*	208-381	0.548	0.01-0.02
10/6/38	20*	258-419	0.232	> 0.1
6/7/38	20*	257-418	0.699	< 0.001
Combined estimate of <i>r</i> for 58 birds = 0.515, <i>P</i> < 0.001				
(b) Total dose = 18.0 mg. AP52C (Approx. 16 I.U.)				
23/2/38	20†	260-412	0.487	0.02-0.05
8/3/38	19†	200-406	0.713	< 0.001
17/3/38	16†	235-438	0.404	> 0.1
29/3/38	20*	257-399	0.439	0.05-0.1
7/4/38	20*	257-417	0.412	0.05-0.1
27/4/38	19*	228-407	0.465	0.02-0.05

Combined estimate of *r* for 114 birds = 0.497, *P* < 0.001

* Simultaneous injection of 1.0 ml. sesame oil into each bird.

† Simultaneous injection of 0.5 ml. sesame oil into each bird.

It will be seen that while on many occasions the responses of groups of 'mixed' pigeons to a given dose of hormone were approximately equal, at other times appreciable variations in response have been observed.

Table III. *Mean responses of groups of 'mixed' pigeons to a given dose of prolactin at various times*

Date	Total dose of prolactin AP52C (mg.)	No. of birds	Mean $\frac{\text{crop-weight}}{\text{body-weight}} \times 100$
23/2/38	18	20*	1.401
8/3/38	18	19*	1.420
17/3/38	18	16*	1.579
29/3/38	18	20†	1.355
7/4/38	18	20†	1.427
27/4/38	18	19†	1.361
10/10/39	10.8	14	0.985
13/12/39	10.8	10	0.864
9/2/40	10.8	15	1.168
21/3/40	10.8	10	0.649

* Simultaneous injection of 0.5 ml. sesame oil into each bird.

† Simultaneous injection of 1.0 ml. sesame oil into each bird.

Similar variations which have also been observed in the experiments with the international standard were noted above. One possible cause of such

variations is differences in average age of the birds constituting the groups, since Riddle *et al.* [1933] have found the response of mature birds to a given dose of hormone to be many times greater than that of young birds. For instance, in spring and early summer, batches of birds obtained from dealers might be expected to contain larger proportions of young birds than in the autumn and winter. The age of our birds is quite unknown, and this factor, under our conditions, is uncontrollable. We considered it advisable, however, to investigate other possible sources of variation in the response, and in this connexion factors associated with the season of the year, such as total time of exposure to light, and environmental temperature came to mind.

The effect of light on the crop-gland response

In order to investigate the influence of light, two groups of five pigeons were placed in each of two identical rooms maintained at the same temperature. One room was illuminated throughout the experiment with a 100-watt bulb while the other was kept dark except for 30-minute periods each morning and evening, during which the birds in both rooms were fed and watered. The hormone injections were made during the first 30-minute break. Two dosage levels were studied with preparation AP52C. The results which are given in Table IV indicate that exposure to light has no influence on the crop-gland response.

Table IV. *Effect of light and darkness on the crop-gland response of pigeons injected with prolactin*

Total dose of prolactin AP52C (mg.)	Unilluminated*		Illuminated*	
	No. of pigeons	$\frac{CW}{BW} \times 100$	No. of pigeons	$\frac{CW}{BW} \times 100$
10.8	5	0.854	5	0.874
15.0	5	1.181	5	1.067

* 'Illuminated' signifies that this group of pigeons was flooded with electric light all the time. 'Unilluminated' signifies that this group was kept in darkness except for 1 hour daily when the pigeons were flooded with electric light to encourage feeding.

The effect of environmental temperature on the crop-gland response

In two preliminary experiments (I and II) the responses to the same two dose levels of preparation AP52C were studied on groups of birds housed in rooms maintained at two widely different temperatures (approx. 0° C. and approx. 30° C.) comparable with extremes of severe winter and hot summer.

Four groups of birds were used in each experiment, ten pigeons in each group in the first and fifteen in each in the second. Two groups were maintained at the lower and two at the higher temperature in each

experiment. In both experiments the birds in one group at each temperature received a total of 10.8 mg. AP52C and all members of the other

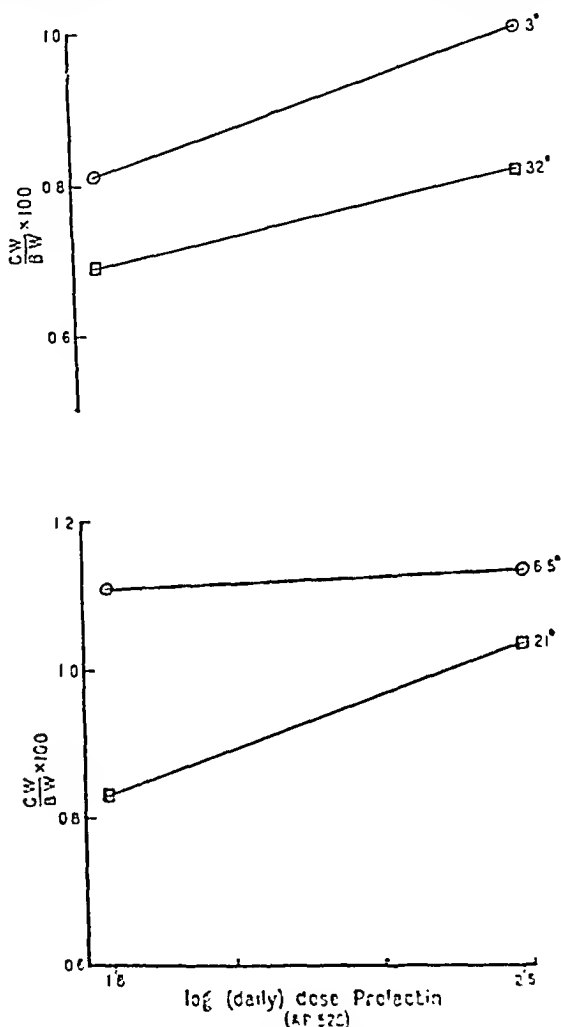


FIG. 4. Effect of temperature on the crop-gland response of pigeons. Curves showing results of two experiments made at two different levels of dosage of prolactin, at a low and a high temperature.

group at each temperature received 15.0 mg. AP52C. The mean temperatures were 0° C. and 21° C. in the first experiment and 6° C. and 32° C. in the second. The results of both experiments are given in Table V and Fig. 4. In both experiments the responses of the birds maintained at the higher temperature were less than of the corresponding birds at the lower

Table V. *Effect of temperature on the crop-weight responses of pigeons to injections of prolactin*

Experiment No.	Date	Temperature (° C.)	Total dose prolactin prepn. (AP52C) (mg.)	No. of pigeons (n)	Crop-glands		Standard error of	t	P
					CW/BW × 100 (g.)	Mean the mean† (e)			
I and II combined	9/1/1940 to 9/2/1940	0-6.5	10.8	(a)* 10	0.380-1.404	0.808	0.05004		P = 0.02 for t = 2.457 for n = 30
				(b)* 15	0.785-1.548	1.108			0.01 " t = 2.750 " n = 30
	21-32	10.8	(a) 10	0.425-1.069	0.689	0.05314	2.5893	P = 0.01 " t = 2.57592 " n = ∞	
				(b) 15	0.510-1.562	0.827			
	0-6.5	15.0	(a) 10	0.688-1.253	1.001	0.05544			
				(b) 15	0.623-1.490	1.122			
21-32	15.0	(a) 10	0.446-1.226	0.817	0.04484	1.9074	P = 0.05 for t = 2.042 for n = 30		
			(b) 15	0.585-1.401	1.034			or " t = 1.95996 " n = ∞	
III	21/3/1940	(i) 4	15.0	10	0.433-1.186	0.649	0.0460	For i/ii 0.8927	P between 0.3 and 0.4
		(ii) 12	15.0	10	0.401-0.841	0.591	0.0458	iii/iv 6.7705	P less than 0.001
		(iii) 21.5	15.0	10	0.397-0.663	0.547	0.0312	i/iv 8.8123	P less than 0.001
		(iv) 31	15.0	10	0.235-0.415	0.334	0.0043	ii/iii 0.7940	P between 0.4 and 0.5
IV	5/4/1940	(i) 4	15.0	15	0.421-2.095	0.891	0.1158	i/ii 0.7031	P between 0.4 and 0.5
		(ii) 10	15.0	15	0.701-1.328	0.981	0.0545	iii/iv 2.5899	P 0.02 at t = 2.532
		(iii) 15	15.0	15	0.465-1.722	1.054	0.0933	iii/i 1.0962	P = 0.3 at t = 1.067
		(iv) 20.5	15.0	15	0.435-1.236	0.773	0.0554	iii/ii 0.6770	P between 0.5 and 0.6
							iv/i 0.9219	P " 0.3 " 0.4	

* (a) refers to an experiment carried out on groups of 10 birds in January 1940. Exp. I.

(b)

† Combined mean for experiments I and II.

15 " February " Exp. II.

temperature, but statistical analysis showed that the significance of the differences was in no case very high. On combining the results of the two experiments, the response to both doses in the temperature range 0° – 6.5° C. was significantly greater than in the temperature range 21° – 32° (see Table V).

These results led us to investigate the possibility that between 0° C. and

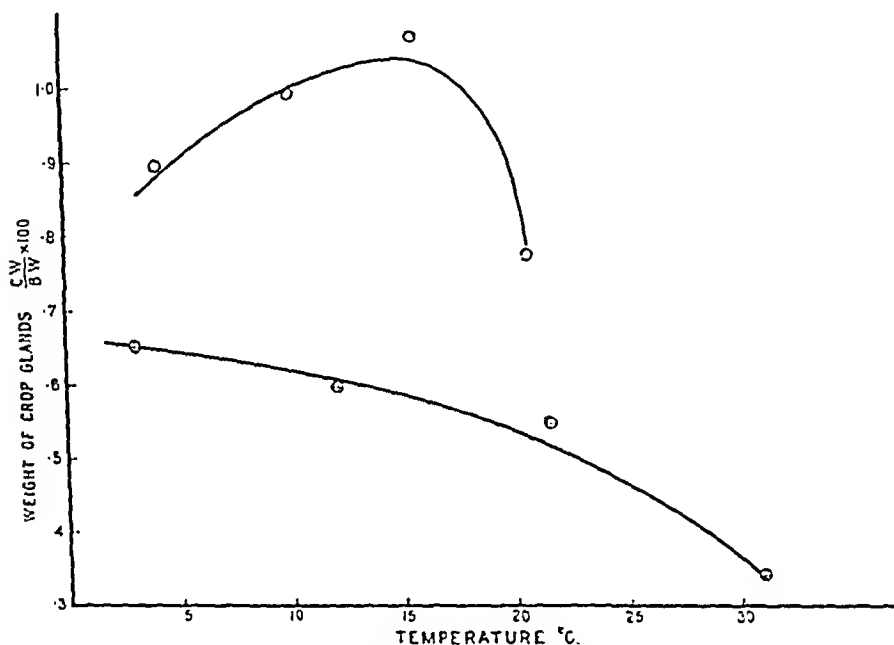


FIG. 5. Effect of temperature on the crop response of pigeons to prolactin. Experiments at four different temperatures.

20° C. there exists an optimum temperature at which a maximum response to a given dose of hormone is obtained. Two further experiments were therefore made in which the response to a single-dose level of AP52C was determined simultaneously in groups of pigeons housed in rooms at four different temperatures. The mean temperatures were 0° , 12° , 21.5° and 31° C. in Experiment III, and 2° , 10° , 15° and 20.5° C. in Experiment IV. The results are given in Table V and plotted in Fig. 5.

In one of these experiments there is an indication of an optimum temperature of maximum response at approximately 15° C., while in the other no optimum is apparent. Statistical analysis of the results (see Table V) confirms the conclusion that the response at temperatures above 20° C. is less than at lower temperatures, and the results of these two experiments indicate that for maximum response the birds should be

preferably maintained at an equable temperature in the region of 15° C. Results obtained with a given preparation at different times of the year, particularly in countries which experience extremes of temperature in winter and summer, are clearly not comparable, and as temperature may not be the only factor influencing the response to a given dose, direct comparisons with a standard should always be made.

Secretory response of the crop-gland to prolactin

It has long been known that the enlarged crop-glands of incubating pigeons produce a nutritive secretion generally, and rather unfortunately, called 'crop-milk'. Riddle & Braucher [1931] showed that this secretion could be elicited in non-incubating pigeons by the injection of anterior pituitary extracts. Early in our experience with the crop-gland test we noted that although many experimentally stimulated crop-glands were coated on the inside with 'crop-milk' this was not invariably true. It seemed possible that only the higher levels of crop stimulation were accompanied by the secretion of large and easily visible amounts of 'crop-milk'. To investigate the relationship between the naked-eye estimate of the crop secretion and the crop-weight and/or hormone dosage, semi-quantitative observations on the gross appearance of the crop secretion in every pigeon have recently been made as a matter of routine.

Four grades of secretion were recognized. Crops in which there was no visible secretion adhering to the crop mucosa are designated as 0; the next stage, +, is characterized by a thin uniform layer of slimy, yellowish secretion extremely difficult to remove; ++ designates the stage in which there is a moderately thick layer of white solidified secretion fairly uniformly distributed over the mucosa, while in the final stage, +++, there is a solid secretion distributed over the mucosa in large discrete lumps quite easy to remove. The observations obtained in the course of the construction of the dose-response curve with the international standard preparation are summarized in Table VI. It will be seen that the amount of secretion which adheres to the crop mucosa is correlated with the dosage of hormone. When the results are plotted (Fig. 6) a sigmoid dose-response curve is obtained. These subjective observations may well form the basis for a method of assay, but it is unlikely to be as accurate or as useful as one based on an objective criterion such as crop-gland weight.

The foregoing results show that within the dose range over which there is a graded increase in crop-weight the amount of secretion produced by the crop is also graded. This raises the question of whether the increase in crop size which follows prolactin injections can be ascribed chiefly to *hypertrophy* due to distension of the epithelial cells with secretion. The results of Leblond & Allen [1937] and Lahr & Riddle [1938] using the

colchicine technique show, however, that true *hyperplasia* occurs at the same time, so that we may assume the graded increase in crop-weight is due both to multiplication of the epithelial cells and to their secretory activities.

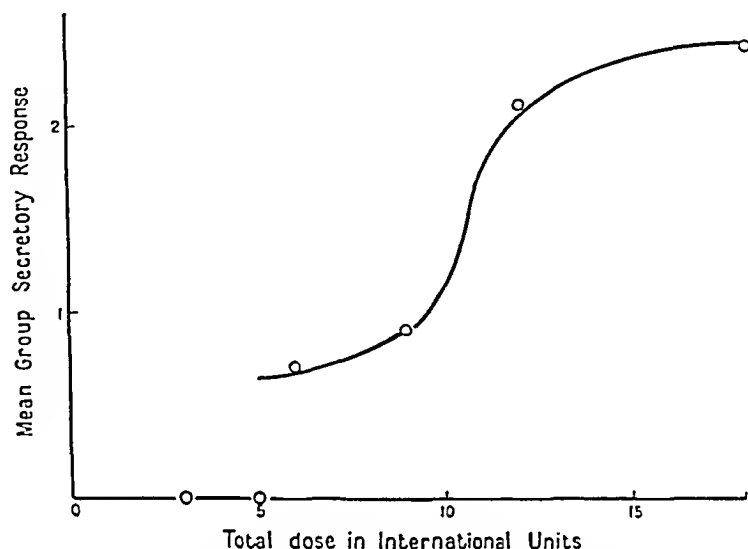


FIG. 6. Crop secretory response to prolactin. Mean quantity of secreted 'Crop milk' (denoted by 0, +, ++, +++) related to dose of prolactin (I.U.).

(Note. A minimum dose of approx. 6 I.U. appears to be necessary to produce a visible secretion.)

Table VI. Graded secretory response of the pigeon crop-gland to different doses of prolactin

Date of assay (1st injection)	No. of pigeons in group	Total dose (I.U.)	Distribution of crop-glands according to degree of response				Mean secretory response of group
			0	+	++	+++	
6/2/40	10	3	10	—	—	—	0
27/2/40	7	5	7	—	—	—	0
6/2/40	10	6	5	3	2	—	+0.7
27/2/40	7	9	3	2	2	—	+0.2
6/2/40	9	12	—	1	6	2	+2.1
6/2/40	10	18	—	1	4	5	+2.4

SUMMARY

1. The results of numerous prolactin assays by the pigeon crop-weight method have been analysed statistically.

2. The conclusions reached are:

(a) There is a positive correlation between crop-weight and body-weight, for a given dose of hormone.

- (b) A sigmoid curve is obtained when either absolute crop-weight or crop-weight expressed as a percentage of body-weight is plotted against dose of hormone. The relationship between either of the above quantities and log dose is approximately rectilinear for total doses from 3 to 18 I.U.
- (c) If certain conditions are fulfilled, the accuracy of the method is of the same order as that of the more precise biological methods of assay described in the British Pharmacopoeia [1932] and the Addendum [1936].
- (d) Light has no influence upon the response, but temperatures above 15° C. decrease the stimulation of the crop-glands by prolactin.

3. At autopsy a subjective estimate of the 'crop-milk' adhering to the glands of pigeons treated with prolactin shows a graded response between the dose and the amount of secretion. This may afford the basis of an approximate assay method, empirical and less objective than methods based on the weighing of glands.

4. To attain a satisfactory degree of accuracy in assays of prolactin the following conditions should be observed:

- (a) Each group should consist of 15-20 birds.
- (b) All the birds should be kept at the same temperature, preferably near 15° C.
- (c) The body-weights of the pigeons should lie within the limits 260-360 g., the mean weights of each group being as nearly equal as possible.
- (d) The hormone injections should be subcutaneous, carefully avoiding chance intramuscular or intradermal injections which produce less response.
- (e) Simultaneous comparison between the preparation under test and the standard preparation of prolactin should be made, and the results interpreted by reference to a curve relating dose to response, previously constructed in the laboratory making the assay.
- (f) The calculations should be made on the basis of crop-weights expressed as percentages of body-weights.

We are indebted to Dr. F. G. Young for the gift of the preparation AP52C and to Messrs. S. C. Watson and A. C. Quiney for technical assistance.

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THE DOSE/RESPONSE RELATION FOR CERTAIN PRINCIPLES OF THE PITUITARY GLAND, AND OF THE SERUM AND URINE OF PREGNANCY

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NOTATION

- x , The dose of a preparation injected into an animal.
 t , Time.
 y , Any variable dependent upon x or t .
 \bar{y} , The mean of a group of determinations of y at a given value of x .
 α , The standard interval (see p. 199) of a logistic curve.
 β , The distance of the point of inflexion of a logistic curve from the origin.
 Y , The number of standard intervals equivalent to y (see p. 222).
 X , x/α , the dose when the standard interval is taken as unity.
 B , β/α , the distance of the point of inflexion from the origin when the standard interval is taken as unity.
 X_0 , $(\beta-x)/\alpha$, or $B-X$.
 τ , $(\beta-t)/\alpha$, analogous with X_0 .
 L , The limiting value of y in a logistic curve.
 y_p , \bar{y}/L .
 w , Any weight factor.
 n , The number of animals in a group.
 σ_y , $\sigma_{\bar{y}}$, etc., The standard deviation of y , \bar{y} , &c.
 $S(-)$, The sum of all values of any expression in the bracket.
 y' , x' , &c., Any given value of y , x , &c.

INTRODUCTION

DOSE/RESPONSE curves are usually determined for the purposes of biological assay, and for this reason the part of such curves receiving practical consideration is the steep, central portion. When it is desirable to express the potency of one preparation in terms of that of another possessing similar biological activity, any mathematical form which fits the data within the limits of random sampling may be used. Moreover, the range over which assays are carried out may be restricted so that any particular form, chosen for its simplicity or for other advantages, fits the data within prescribed limits, or that form may yet be used even when there is more scatter than would be expected from random sampling, and the excess taken into account in the estimation of errors.

When dealing with graded responses, in which the response is a continuous variate, the form $y = a + b \log x$, where y is the response and x the dose, possesses peculiar advantages. A satisfactory method exists for fitting the curve, by which the errors of the constants, and hence of the resulting potency ratios, may be calculated accurately. The curve is independent of the scale of measurement of dosage, and is a straight line when the response is plotted against the logarithm of the dose. A change of scale merely shifts this line parallel to its former position, and if such straight lines are fitted separately to two preparations, the horizontal distance between them is a measure of the logarithm of the potency ratio.

Discussions of dose/response relations have thus been almost wholly confined to the use of this very convenient method of graduation. In two recent communications, Bliss & Marks [1939 *a, b*] have given detailed consideration to the design and analysis of experiments involving graded responses, and have evolved useful criteria by which to assess the accuracy with which data are fitted by straight log-dose/response lines. There are, therefore, very good reasons for using curves of the form $y = a + b \log x$ for assay purposes whenever possible. However, there are instances where the use of such a function appears to limit unduly the range of responses which may be used in assaying preparations, with a consequent danger of loss of time and material when a preparation of unknown potency is assayed. If it proved possible to handle with ease curves which fit a greater part of the range of dosage, these might frequently be preferred in practice.

It is perhaps unnecessary to stress the fact that a curve of the form discussed above cannot fit the whole dose range. It usually fits well enough over that range of dosage where the response is changing most rapidly with changes of dose, but so do many other forms. As an instance [Emmens, 1939], the response of the capon's comb to androsterone was about equally well related to the dose by the following diverse equations:

$$y = 0.57x + 2.65,$$

$$y^2 = 7.2x,$$

and

$$y = 12.48 \log x - 3.07,$$

where x is the total dose and y the response in mm. comb growth. The logarithmic form does not pass through zero response at zero dose, and unless a is made a variable, certain doses must be supposed to elicit negative responses. Furthermore, a curve of this form imposes no upper limit on the response.

It should be noted that the foregoing does not apply to a discussion of quantal (discontinuous) responses. When dealing with these, it is common to use the normal equivalent deviation [cf. Gaddum, 1933] or probit, which is formed by adding 5 to the normal equivalent deviation, and to plot it against the logarithm of the dose. The rationale of such a procedure depends on the observation that the logarithms of the individual effective doses tend to be normally distributed more often than are the doses themselves. The resulting mathematical relationship is therefore different from that under discussion.

In the present communication we shall consider another way of relating dose to effect by a type of curve which, in the cases discussed, follows the whole course of the response. The argument concerns primarily the nature of the dose/response curves rather than their adaptability for assaying preparations, although a certain amount of discussion is included on the subject of errors and relative potencies. It is my intention to give further consideration at a later date to the practical use of the curves adopted.

No simple dose/response curve is likely to reflect with complete accuracy the response of an organ or of an individual to various doses of a pharmacologically active agent. Changes in the type or mechanism of response which may often be expected at different dose levels will cause deviations from a simple curve, apart from considerations of individual variation. Thus, it is doubtful whether any such curve holds good over an entire dose range, unaffected by compensatory or other mechanisms which will, in all probability, rarely act in a completely regular or simple manner. However, it seemed possible to find 'rational' curves which fit certain sets of data better than those hitherto discussed in connexion with them, and which might have a rather higher status than that of an avowedly convenient graduation. An attempt is made to do this, using the response of the ovary of the immature rat to gonadotrophins from various sources, that of the guinea-pig thyroid gland to thyrotrophin and of the pigeon crop-gland to prolactin. These all involve the increase in weight of the organs concerned under the appropriate stimulation, and are thus more simple and natural measures of the effect of an active agent than tests in which an

arbitrary index of response is used, or in which a definitely indirect action is measured. They may therefore be expected to give curves which reflect the course of the response of an organ in a way as little artificial as we could hope to employ.

It is appropriate here to record my indebtedness to the work of Chou & Liu [1937-8], whose paper on the assay of certain gonadotrophins contains the only reference I have found to the use in such assays of the type of curve, the logistic, I am about to discuss. Their use of this curve was a stimulus to investigate it further, and their conclusions will be discussed later in relation to my own.

THE LOGISTIC CURVE

Towards the middle of the last century Verhulst [1838, 1845, 1847] published a series of memoirs on the growth of population, concluding that it could be expressed by a curve of the following type:

$$y = \frac{L}{1 + e^{(\beta - \alpha)t}}, \quad . \quad . \quad . \quad . \quad (1)$$

when y is the population, t the time and L the limiting value of the population in a given area. The constants α and β determine the scale of the curve.

Ostwald [cf. 1883 *a, b*] used the curve to describe autocatalytic reactions, and Robertson [cf. 1908 *a, b*] first applied it to the growth of the individual and to that of organs. Robertson used the form:

$$\log \frac{x}{A-x} = K(t-t_1), \quad . \quad . \quad . \quad . \quad (2)$$

where A is the final weight or volume attained, x the weight at any given time, t the time at which growth is half completed and K a constant. Equation (2) may also be expressed as:

$$x = \frac{Ae^{K(t-t_1)}}{1 + e^{K(t-t_1)}}, \quad . \quad . \quad . \quad . \quad (3)$$

Pearl & Reed [1920], independently of Verhulst's earlier work, re-investigated the growth of population and again arrived at a logistic formula, using an equation similar to (3) above. Later, Reed & Berkson [1929] discussed general applications of the form:

$$y-d = \frac{K}{1 + Ce^{rt}}, \quad . \quad . \quad . \quad . \quad (4)$$

where d , K , C and r are constants, and the lines $y = K$ and $y = d$ are asymptotes of the curve. They also discussed methods of fitting the curve

instead of a time, t , we see that [cf. Yule, 1925]

$$\frac{1}{y} \frac{dy}{dx} = \frac{1}{\alpha} \left(1 - \frac{y}{L} \right) \quad . \quad . \quad . \quad . \quad . \quad (7)$$

and

$$\frac{d^2y}{dx^2} = \frac{1}{\alpha} \left(1 - \frac{2y}{L} \right) \frac{dy}{dx} \quad . \quad . \quad . \quad . \quad . \quad (8)$$

The constant of integration, β , is evidently to be measured in the same units as x . By analogy with the standard deviation, α was termed by Yule the 'standard interval', a name which we shall adopt here. The point of inflexion of the curve, given by (8), is $y = \frac{1}{2}L$, $x = \beta$. The curve, symmetrical about the point of inflexion, is furthermore asymptotic to $y = 0$, $y = L$. Following Yule's simplification (5), we may write:

$$y_p = \frac{1}{1 + e^{-X_0}}, \quad . \quad . \quad . \quad . \quad . \quad (9)$$

$$\frac{1}{y_p} \frac{dy_p}{dX_0} = 1 - y_p, \quad . \quad . \quad . \quad . \quad . \quad (10)$$

where $y_p = y/L$ and $X_0 = (\beta - x)/\alpha$, and X_0 is measured with the standard interval as a unit, the point of inflexion being $X_0 = 0$. The proportional rate of increase of y is a linear function of y (equation (10)) and falls continuously from the start.

Yule [1925] gives tables of the corresponding values of y and τ (X_0 in our case) for equations (5) or (9), and points out that we could draw a logistic curve once and for all and fit the data to it by replacing the actual value of y by its ratio to the limiting value, L , making the points of inflexion coincide, and taking the standard interval as unity. This is done in the curves which follow, except that the actual values of y have been indicated on the appropriate axes. Curves which share the same value of L can then be included on a single graph by adjusting the horizontal scale in each case.

These considerations help us to see why a function such as the logistic should be applicable to dose/response relationships in which the growth of an organ is involved. Such growth has both an upper and a lower limit, and the proportional response falls as the total dose of a trophic substance rises. Although the latter condition is also fulfilled by a linear log-dose/response relationship, the former is not. Further discussion of the factors which may be supposed to determine the logistic nature of the dose/response curve are best left until we have demonstrated its practical applicability, and we shall consider them in some detail later on.

METHODS OF FITTING THE CURVE

A number of methods of fitting the logistic curve have already been discussed by Verhulst, by Pearl & Reed [1920], Yule [1925], Reed &

Berkson [1929] and Wilson & Puffer [1933]. They are mostly applicable with success to data which run smoothly and to which a curve could be fitted by eye alone with little error. The deviations of our own data from any curve which may be fitted to them are often large, and the error of any given determination may differ widely from that of another. The only method already described which seemed likely to be useful to us was that of percentage increases (Method (3) of Yule [1925]), and the corresponding method of Reed & Berkson [1929] by which $\log\{(L-y)/y\}$ should lie on a straight line if plotted against x . The methods were found to be tedious and not very accurate in practice, and the following method was finally adopted as giving with fair accuracy the best fit of a series of points to a previously chosen curve.

In the first place, a method of trial and error confirmed my belief that the dose/response curve should have, as its lower asymptote, the x axis, and not as an asymptote a line parallel to that axis and passing through the control weight, as was used by Chou & Liu [1937-8], with, however, certain departures from their theory. Secondly, the upper asymptote is determined from those points which are obviously on the 'ceiling' of the curve. It was sometimes necessary to make a few trials in order to determine the best value for this asymptote, and in grouping results with similar extracts on one curve an approximate value has been found sufficient for our purpose.

The scale of the y axis being determined, the curve is then drawn to cut the y axis at the control weight and with standard intervals marked out along the x axis, or a standard curve may be used with suitable adjustment of the axes and scales. Those points which were not used to form an estimate of the limiting value of y are then used to read off from the curve the number of standard intervals equivalent to each mean response, \bar{y} , taking the origin as 0. Alternatively, the entire process may be carried out mathematically or by the use of Yule's tables, but the more rapid graphical method seems sufficiently accurate. Each total dose is then divided by the corresponding number of standard intervals, giving a series of estimates of the number of mg. or units of extract equivalent to a standard interval. A mean value of this latter quantity is then taken to determine the final dose scale, and the points are plotted on that scale. If the variance of the mean proportional response (\bar{y}'/L) varies from group to group, these estimates may be weighted accordingly, but the percentage standard error of the response is often fairly constant.

This relatively simple and rapid method yields satisfactory results. If it were of great importance to determine with exactitude the best-fitting logistic curve, additional refinements of the method would be necessary, but as it stands, it gives a good approximation to the best-fitting curve,

quite adequate for dealing with the type of data with which we are concerned below. For accurate working, it must be recalled that σ_α , the standard error of any given estimate of α , is not directly proportional to σ_y , and that the estimates of L and of the control weight are subject to error.

Considering the form
$$y_p = \frac{1}{1 + e^{(\beta - x)/\alpha}},$$

let y_{p0} ($= \bar{y}_0/L$) be the value which corresponds with $x = 0$.

Then
$$y_{p0} = \frac{1}{1 + e^{\beta/\alpha}}.$$

Hence

$$\frac{\alpha}{x} = \frac{1}{\log_e\left(\frac{1}{y_{p0}} - 1\right) - \log_e\left(\frac{1}{y_p} - 1\right)},$$

$$\sigma_\alpha = \frac{\alpha^2}{x} \sqrt{\left(\frac{\sigma_{y_{p0}}^2}{y_{p0}^2(1-y_{p0})^2} + \frac{\sigma_{y_p}^2}{y_p^2(1-y_p)^2}\right)},$$

Where

$$\sigma_{y_{p0}}^2 = \frac{\sigma_y^2}{L^2} + \frac{\bar{y}_0^2 \sigma_L^2}{L^4},$$

$$\sigma_y^2 = \frac{\sigma_y^2}{L^2} + \frac{\bar{y}^2 \sigma_L^2}{L^4}.$$

The standard errors above apply to large samples only.

If we ignore the error of y_{p0} , which is small when an adequate number of controls have been used, and that of L , the value of which may be determined quite accurately if necessary, we find

$$\sigma_\alpha = \frac{\alpha^2}{x} \cdot \frac{\sigma_{y_p}}{y_p(1-y_p)}.$$

If the percentage standard error is constant, i.e. $\sigma_{y_p} = ky_p$,

$$\sigma_\alpha \propto \frac{\alpha^2}{x(1-y_p)}.$$

The weights to be used in the calculations may be constructed so that

$w' \propto \frac{1}{\sigma_\alpha^2}$ in each case.

In practice, we do not know the error of L , and unless we devote considerable time and labour to reducing this error to negligible proportions by the addition of sufficient experimental data, the exact error of α probably cannot be determined in the way indicated above. On page 222, consideration is given to a further and probably more profitable method of fitting the logistic curve when such errors may be of importance, as in the assay of one preparation in terms of another. The present method of fitting is, however, more rapid, and adequate for its purpose.

Example

We take as an example the assay of PMS22 (Table I), an extract of pregnant mares' serum, which, when injected into immature female rats, causes an increase in ovarian weight.

Table I. *Data relating to the fitting of PMS22*

Total dose (x') in mg.	Wt. of ovaries (\bar{y}') in mg.	Equiv. dose in standard intervals (X')	Weight factor (w')	$\frac{x'}{\bar{X}'}$	$\frac{w'x'}{\bar{X}'}$
0.01	23	0.86	80	0.0113	0.908
0.02	53	1.84	230	0.0107	2.465
0.03	65	2.12	447	0.0141	6.296
0.05	172	4.27	119	0.0117	1.392
Sum:	—	—	876	0.0478	11.061

From this and similar extracts, the limiting response has been found to be approximately 220 mg. From the curve, $y = 220/1 + e^{-x}$ and the line

Table II. *Data relating to the response of the ovary of the immature rat to extracts of the serum of pregnant mares*

Extract	Total dose (mg.)	No. of rats	Wt. of ovaries (mg.)	No. of mg. equivalent to a standard interval
International standard preparation	2.5	10	22	4.08
	5.0	"	33	
	7.5	"	50	
	10.0	"	79	
	15.0	"	141	
	20.0	"	194	
	25.0	"	202	
PMS14	1.5	10	20	3.03
	2.5	"	25	
	5.0	"	47	
	10.0	"	106	
	25.0	"	168	
PMS18	0.5	20	42	0.345
	1.0	15	104	
	1.5	10	163	
	2.0	"	206	
	2.5	"	245	
PMS22	0.01	10	23	0.0126
	0.02	9	53	
	0.03	10	65	
	0.05	5	172	
PMS24	0.25	20	33	0.208
	0.375	10	56	
	0.5	20	91	
	0.75	10	131	
	1.0	"	203	
	5.0	5	178	

$X = -2.99$, which cuts the curve at the point $y = 10.6$, the control ovarian weight, we determine the data in column 3 of Table I. The weight factor, w , is calculated as indicated above, the quantity $S(w'x'/X')$ divided by $S(w')$ gives the mean number of mg. of PMS22 equivalent to a standard interval, this is 0.0126. If weight factors are not used, it works out very nearly the same, at 0.0120. The data for PMS22 are then plotted on the curve (Fig. 2) with $\alpha = 0.0126$ mg., due allowance being made for the sign of X_0 , which is used in the figures so that it has zero value at the point of inflexion of the curve, although the actual dose naturally has zero value at the origin. This change of scale, although perhaps confusing at first, has been adhered to because it remains a constant measure from curve to curve, whatever the nature of the response investigated or the extract causing it, and enables one to see at once the relation of the point of inflexion to the y axis. If equation (9) be written in the form:

$$y = \frac{L}{1 + e^{B-X}}, \quad . \quad . \quad . \quad . \quad . \quad (11)$$

where $B - X = X_0$, then in the example, $L = 220$ as before, $B = 2.99$ (the distance of the origin from the point of inflexion when reading along the x axis), and $X = x/0.0126$. This is the most convenient form in which to write the equations of the curves which follow.

RESPONSE OF THE OVARY OF THE IMMATURE RAT TO GONADOTROPHINS

The technique of assay used in determining the activity of gonadotrophins has been described by Deanesly [1935]. Groups of 10 rats per dose are usually employed, at a body-weight of between 40 and 50 g. An injection is given each day, for 5 consecutive days, the animals are killed on the sixth day and the ovarian weight is determined after fixation in Bouin's fluid and immersion in 70% alcohol.

(a) *Extracts of the serum of pregnant mares*

Five preparations of the serum of pregnant mares have been investigated in sufficient detail to be used to form an estimate of the nature of the dose/response curve (Table II and Fig. 2). The average ovarian weight of 100 unstimulated immature rats of the body-weight used in the tests was 10.6 mg. This may be taken as the point on the y axis through which the dose/response curve must pass. As described above, the maximum average ovarian weight obtainable under stimulation from this type of extract is approximately 220 mg., which is taken as the limiting value of y . The data were therefore plotted in relation to the curve,

$$y = \frac{220}{1 + e^{2.99 - X}}$$

The dotted curve in Fig. 2 is logarithmic, and has the equation

$$y = 381.0 \log X - 72.1.$$

It has been fitted to the central values, between $X_0 = -1$ and 4, which values it fits very well. But, it is clearly impossible to obtain a good fit with such a curve over the whole range of response, whereas the logistic curve covers this complete range. Although the data for different extracts have been made to fall on the same logistic curve, by adjusting the abscissae, this has little to do with the departure shown from the logarithmic curve, as no amount of adjustment would give a good fit to the latter. An extrapolation of the logistic curve to the left of the y axis may not be without meaning. The relationship between the dose/response curve and the growth which is occurring as a result of the natural stimulation from the animal's own gland(s) will be discussed later, and it is sufficient for the moment to indicate that the logistic curve may have a meaning throughout more than the range covered by experimental findings.

(b) *Extracts of human and of horse pituitary glands*

One preparation of human pituitary glands from both sexes, two of horse pituitary glands and one of gelding pituitary glands have been investigated and have been found to have, within narrow limits, the same dose/response curve. This curve, shown in Fig. 3, plotted from the data of Table III, is a logistic curve with an L of 100, having the equation

$$y = \frac{100}{1 + e^{2.13 - X}}.$$

Table III. *Data relating to the response of the ovary of the immature rat to extracts of human and horse pituitaries*

Extract	Total dose (mg.)	No. of rats	Wt. of ovaries (mg.)	No. of mg. equivalent to a standard interval
AP47 (Human)	1.0	10	22	1.031
	2.0	..	49	
	3.0	..	66	
	4.0	..	84	
	5.0	5	100	
AP41D (Horse)	0.5	10	33	0.483
	1.0	..	44	
	2.5	5	78	
AP61B (Horse)	0.5	5	15	1.080
	1.25	10	25	
	2.5	..	58	
	5.0	..	95	
	10.0	5	99	
AP70B (Gelding)	1.25	5	29	1.016
	2.5	10	56	
	5.0	5	97	

A logarithmic curve fitted to the same points, the dotted curve in Fig. 3, is again an excellent fit over a limited range, with wide departures from the true curve at the upper and lower limits of response. The equation of this curve is

$$y = 130.8 \log X + 8.24.$$

Extracts of sheep, pig or ox pituitary glands do not produce such high maximal responses as those considered above. Our data with regard to these extracts are not sufficiently extensive to warrant the plotting of

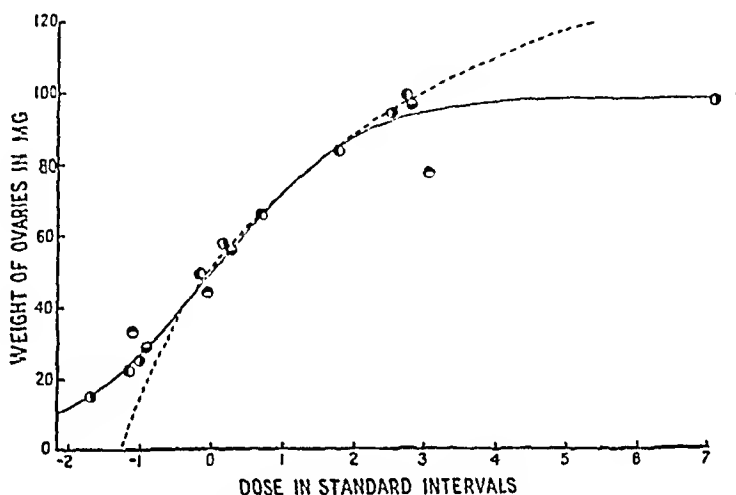


FIG. 3. The logistic curve fitted to the data for the response of the ovary of the immature rat to extracts of pituitary glands (Table III). The dotted curve is a logarithmic curve (see text) which fits the data over a wide but limited range.

- | | |
|------------------|--------------------|
| ○ AP47 (Human) | ● AP41 D (Horse) |
| ○ AP61 B (Horse) | ● AP70 B (Gelding) |

curves, but the highest ovarian weights attainable are approximately as follows: pig and sheep pituitaries 25–30 mg.; ox pituitaries 15–20 mg. A ceiling of 100 mg. is thus by no means characteristic of pituitary preparations.

(c) *Extracts of the serum and of the urine of pregnant women*

Table IV and Fig. 4 show the data available for three extracts, one from the serum and two from the urine of pregnant women. The response of the rat ovary to extracts from these sources is very limited, not exceeding 40 mg. with urinary extracts, and 50–60 mg. with extract of the serum.

The serum extract, PWS77, provides the only clear-cut case in which the logistic curve cannot be said to fit the data better than a logarithmic one (see p. 214). The urinary extracts are fitted well by a curve asymptotic to $y = 40$, the equation of which is:

$$y = \frac{40}{1 + e^{1.03 - X}}.$$

Table IV. *Data relating to the response of the ovary of the immature rat to extracts of the serum and urine of pregnant women (chorionic gonadotrophin)*

Extract	Total dose (mg.)	No. of rats	Wt. of ovaries (mg.)	No. of mg. equivalent to a standard interval
PWS77 (serum)	0.0625	10	13	0.139
	0.125	19	21	
	0.1875	10	23	
	0.25	"	27	
	0.5	"	32	
	0.85	20	37	
	1.25	10	43	
	2.0	"	47	
	2.5	"	57	
	5.0	"	51	
UP12 (urine)	0.0625	5	20	0.0725
	0.125	"	27	
	0.25	"	34	
UP27 (urine)	0.0125	10	14	0.0278
	0.025	15	19	
	0.05	20	27	
	0.1	10	40	
Unstimulated controls for Tables II-IV	—	100	10.6	—

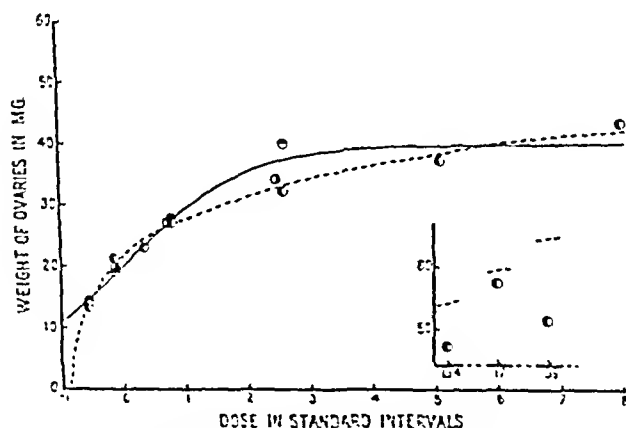


FIG. 4. The logistic curve fitted to the data for the response of the ovary of the immature rat to extracts of the serum or urine of pregnant women (Table IV). The dotted curve is a logarithmic curve which fits the data for PWS77 better than does the logistic curve.

- PWS77 (Serum extract)
- UP12 (Urine extract)
- UP27 (Urine extract)

The serum extract is fitted well by the logarithmic curve

$$y = 21.03 \log X + 21.50$$

over the range $y = 10$ to 40 mg., but not so well beyond that. A logistic curve asymptotic to a higher ovarian weight fits the data for PWS77 even worse than the curve asymptotic to 40 mg.

It will be seen that with these extracts, which give a low 'ceiling' value for the ovarian weight, the sigmoid character of the logistic curve is masked, when only that part which lies to the right of the y axis is considered. This masking is, as we have seen, accompanied in the cases under consideration by a less definite superiority of the logistic curve over a logarithmic one.

RESPONSE OF THE THYROID GLAND OF THE GUINEA-PIG TO THYROTROPHIN

Eight thyrotrophic extracts of ox pituitary glands have been tested by the method of Rowlands & Parkes [1934], by which female guinea-pigs of about 200 g. body-weight are given a daily injection on each of five consecutive days, and are killed on the sixth day. The thyroid glands are fixed in Bouin's solution and weighed after a period in 70% alcohol. Rowlands & Parkes originally concluded (using extract T17B) that the thyroid weight bears a linear relationship to the logarithm of the dose. The fuller data now available (Table V and Fig. 5) show that a logistic curve is to be preferred for descriptive purposes. This curve, passing through the control thyroid gland weight of 30 mg., is asymptotic to a limit of approximately 90 mg. and has the equation

$$y = \frac{90}{1 + e^{0.69 - X}}.$$

The logarithmic curve,

$$y = 66.10 \log X + 50.0,$$

is almost coincident with the logistic one over the range used for practical purposes, but it does not, of course, pass through the necessary control value, and rises too steeply above a thyroid gland weight of 80 mg.

A peculiarity of the response of the guinea-pig's thyroid gland to these extracts is that the standard error of the mean weight of glands from a group of animals varies little at different dose levels. Hence, the scatter of the points about the curve is as great near the control weight as it is at higher values.

RESPONSE OF THE PIGEON CROP-GLAND TO PROLACTIN

Nine preparations of prolactin from the anterior pituitary glands of oxen or pigs (Table VI) have been assayed by the method of Rowlands [1937].

Briefly, daily injections are given to male or female pigeons on six consecutive days, and the birds are killed and the crop-glands dissected on the seventh day. The weight of the crop-gland is determined after fixation in Bouin's fluid and immersion in 70% alcohol. In his original method,

Table V. *Data relating to the response of the guinea-pig thyroid glands to extracts of ox anterior pituitary glands*

Extract	Total dose (mg.)	No. of guinea pigs	Wt. of thyroids (mg.)	No. of mg. equivalent to a standard interval
AP8B	7.5	10	52	10.42
	15.0	"	58	
	20.0	"	70	
	35.0	"	79	
T17B	10.0	10	54	11.77
	20.0	"	64	
	35.0	"	80	
	50.0	"	83	
AP32D1	2.5	5	52	3.05
	5.0	35	66	
	10.0	5	79	
	20.0	"	92	
AP15B	10.0	25	47	11.36
	20.0	15	67	
	35.0	10	83	
P22B	10.0	10	50	8.33
	20.0	"	82	
	35.0	"	85	
AP37D1	5.0	10	45	3.85
	7.5	"	80	
	10.0	"	86	
AP32D1	3.25	5	50	4.19
	5.0	15	54	
	10.0	10	77	
TH10	0.75	10	43	0.877
	1.25	"	66	
	2.5	"	81	
Controls	—	25	30	—

Rowlands expressed the weight of the crop-glands as a percentage of the body-weight, but the crude crop-gland weights have been found rather more satisfactory in this particular investigation. The average weight of thirty unstimulated crop-glands was 1.31 g.

The data for the first three extracts listed in Table VI are fitted by a logistic curve with an upper limiting value of $y = 10$ g. (Fig. 6) having the equation:

$$y = \frac{10}{1 + e^{-x}}$$

and less well by the logarithmic function,

$$y = 7.754 \log X + 3.21,$$

which is the best-fitting log-dose/response curve.

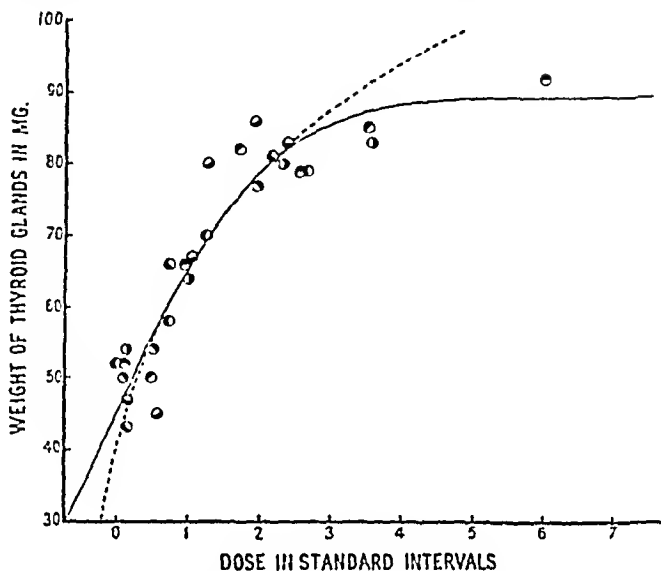


FIG. 5. The logistic curve fitted to the data for the response of the thyroid gland of guinea-pigs to various thyrotrophic (pituitary) extracts (Table V). The dotted curve is a logarithmic curve (see text), which fits the data over a wide, but limited, range.

- | | |
|----------|----------|
| ● APSB | ● P22B |
| ● T17B | ● AP37D1 |
| ● AP52D1 | ● AP32D1 |
| ● AP15B | ● TH10 |

The remaining six extracts are fitted by a logistic curve with an upper limiting value of $y = 7$ g. (Fig. 7), having the equation

$$y = \frac{7}{1 + e^{1.47 - X}},$$

and markedly less well by the logarithmic curve

$$y = 6.381 \log X + 2.51,$$

which fits the data quite well over the range $X_0 = -0.5$ to $X_0 = 3$, but not beyond these limits. It is again clear that a logarithmic curve cannot describe the whole range of the response to such extracts.

We are unable to explain why these lactogenic preparations segregate into two groups. In common with all of the assays described here, they were made over a long period, usually only a point or two being determined at a time. This segregation, which cannot therefore be ascribed to time-to-time variation in the sensitivity of the birds used, or to assay technique, is seemingly due to differences in the properties of the extracts.

Table VI. *Data relating to the response of the crop-gland of pigeons to extracts of anterior pituitary glands (prolactin)*

Extract	Total dose (mg.)	No. of pigeons	Wt. of crop-glands (g.)	Crop-gland wt. ÷ body wt. %	No. of mg. equivalent to a standard interval
AP37C (ox)	3.0	20	1.70	0.52	6.00*
	4.5	"	2.12	0.67	
	6.0	"	4.26	1.13	
	10.5	"	4.45	1.35	
	18.0	"	7.23	2.12	
	30.0	10	9.20	2.25	
	52.8	"	10.30	2.36	
AP50C (ox)	6.0	10	2.22	0.65	10.10*
	12.0	20	3.02	1.00	
	18.0	10	4.21	1.46	
	24.0	20	4.86	1.41	
AP55C (ox)	3.0	10	3.82	1.12	2.61*
	6.0	"	5.45	1.56	
	18.0	"	9.31	2.38	
AP48C (ox)	3.0	5	4.06	1.22	1.56†
	6.0	10	6.53	1.94	
	18.0	"	7.55	2.07	
LP8 (ox)	0.6	10	1.35	0.46	1.46†
	1.5	"	2.84	0.89	
	3.0	5	5.17	1.35	
	6.0	"	6.18	1.60	
AP62 (?)	1.5	5	1.21	0.28	3.87†
	3.9	10	2.71	0.69	
	6.0	5	4.35	1.54	
	12.0	"	5.19	1.34	
	30.0	"	6.94	1.94	
AP32C (ox)	3.0	10	4.01	1.07	1.71†
	6.0	5	6.19	1.90	
	12.0	"	6.34	2.12	
AP43C (pig)	18.0	10	1.68	0.59	64.9†
	30.0	5	1.70	0.55	
	60.0	"	2.95	1.01	
LP1 (ox)	0.6	5	2.31	0.56	0.56†
	0.9	10	5.13	1.60	
	1.5	"	6.24	1.61	
	1.8	5	6.50	1.76	
	3.0	"	8.20	1.84	
	6.0	10	5.78	1.35	
Control	--	30	1.31	0.56	--

* Fig. 6.

* Fig. 7.

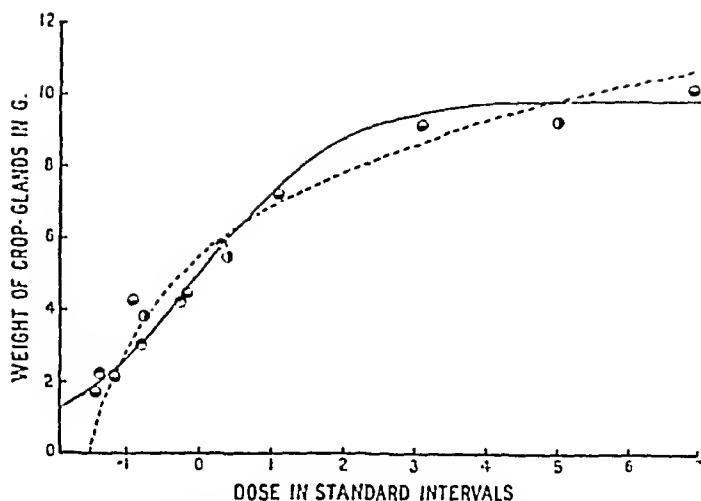


FIG. 6. The logistic curve fitted to the data for the response of the pigeon crop-gland to various lactogenic (pituitary) extracts (Table VI). The dotted curve is a logarithmic curve (see text), which appears to fit the data nearly as well as the logistic curve.

● AP37C ● AP50C ● AP55C

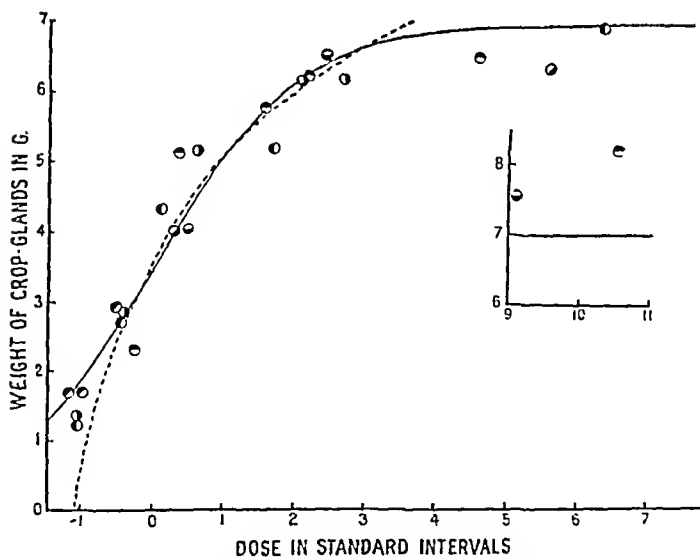


FIG. 7. The logistic curve fitted to the data for the response of the pigeon crop-gland to further lactogenic (pituitary) extracts (Table VI). The dotted curve is a logarithmic curve (see text), which fits the data over a limited range.

● LP8 ● AP48C
● AP62 ● AP43C
● LP1 ● AP32C

GOODNESS OF FIT

The convenient and relatively rapid method of fitting which has been used has, besides the disadvantage that errors are not calculable with accuracy, the further related disadvantage that no exact test is possible of the goodness of fit obtained. The first drawback is of little moment at the present stage, and the second is somewhat annulled by the fact that, even if an exact test of goodness of fit were available, time-to-time variation in response might vitiate much of its supposed accuracy. This source of variation, which is known to occur in many assays of these types, has been almost completely ignored, for in only one instance (UP12) have all of the points been determined simultaneously.

The ratio between the variances

$$\frac{S\left\{n\left(\bar{y}-\frac{L}{1+e^{(\beta-x)\alpha}}\right)^2\right\}}{k-2}$$

and

$$\frac{S(y-\bar{y})^2}{S(n)-k},$$

where n is the number of animals corresponding to dose x , and k is the number of doses, should, however, give a sufficient indication of the accuracy with which the points are fitted by the curves. The variance ratios obtained by this method will not have exactly the distribution upon which Fisher & Yates's [1938] tables are founded, because least squares have not been used, the logistic curve is not linear in α , β and L and the variance of y may not be the same at all values of x . It is interesting, therefore, that the results so obtained (Table VII) appear to give reasonable answers.

The first variance shown above was calculated exactly as indicated in the formula, and is referred to in Table VII as the variance due to deviations from the logistic. The second variance, referred to as that due to random sampling, was not in fact estimated in detail from the full data, but is quite accurately known for each type of test from the following relationships. In tests of gonadotrophic activity, each animal contributes on the average a quantity to the total sum of squares of deviations from \bar{y} which is equal to $0.10\bar{y}^2$, where \bar{y} is the mean of the group to which it belongs. In tests of lactogenic activity the quantity is $0.12\bar{y}^2$ per animal, for in both types of test the variance increases approximately as the square of the response. In tests of thyrotrophic activity, the variance is little affected by the response level, and the sum of squares may be taken as receiving on the average a contribution of 130 per animal, when the response is measured in mg. When, as at present, large numbers of

animals and dose levels are involved, the above relationships may be accepted with confidence.

The variance ratios and the values of P , the probability of equalling or exceeding each ratio by chance, which are given in Table VII, indicate a significant difference in only two cases. It will be seen that all extracts of a similar type were at first tested together, and further tested only if

Table VII. *Tests of goodness of fit for the data of Tables II-VI*

Data for	Variance due to	Sum of squares	Degrees of freedom	Mean square	Variance ratio	P
Extracts of pregnant mares' serum (Table II)	Deviations from logistic	53050	25	2122	1.37	> 0.05
	Random sampling	413420	268	1542		
Pituitary gonadotrophins (Table III)	Deviations from logistic	3110	14	222	0.49	> 0.05
	Random sampling	51650	114	453		
Pregnancy gonadotrophins (human) (Table IV)	Deviations from logistic	5370	15	358	2.93	< 0.001
	Random sampling	20920	172	122		
" (urine extracts only)	Deviations from logistic	140	5	28	0.36	> 0.05
	Random sampling	4940	63	78		
Thyrotrophins (Table V)	Deviations from logistic	6410	25	256	1.79	ca. 0.01
	Random sampling	39000	273	143		
" (omitting AP37D1)	Deviations from logistic	3330	22	151	1.06	> 0.05
	Random sampling	35100	246	142		
Prolactins (Table VI) 1st curvo (Fig. 6)	Deviations from logistic	5820	12	485	1.31	> 0.05
	Random sampling	72610	196	371		
Prolactins (Table VI) 2nd curvo (Fig. 7)	Deviations from logistic	3810	22	239	0.77	> 0.05
	Random sampling	42180	136	310		

a significant departure from a variance ratio of unity were found. The first instance of this is found with gonadotrophins from human pregnancy material (Table IV), where the deviations from the logistic are significantly greater than they should be ($P < 0.001$). This departure is entirely due to PWS77, the serum extract. When the urinary extracts alone are tested, the deviations from the logistic are rather less than might be expected, but not significantly so ($P > 0.05$). The second instance occurring with the thyrotrophins, where $P = \text{ca. } 0.01$, is likewise entirely due to one extract, AP37D1. When the data for this particular extract are deleted, the variance ratio becomes 1.06. The other classes of extract, namely, gonadotrophins from the serum of the pregnant mare, and from human and horse

pituitaries and the lactogenic extracts contain no aberrant members and the variance ratios range about unity without departing significantly from it ($P > 0.05$ in all classes).

We may then be well satisfied by the accuracy with which the logistic curve fits all of the material under discussion, with the exception of the two extracts mentioned. The discrepancy with AP37D1 is not serious, and seven other similar extracts are fitted by the curve, but that with PWS77 is very unlikely to have occurred by chance. The test of goodness of fit we have used seems adequate, to judge by the results it gives, and we must therefore conclude that the data for PWS77 are not fitted best by a logistic curve.

THE POSITION OF THE ORIGIN RELATIVE TO THE x AXIS

Fig. 8 shows the points at which the various curves we have discussed are cut by the y axis, omitting, for clarity, the prolactin curves. In each case, the y axis is so arranged that it is divided by the curve at a point, C , at which the value of y bears the same relation to L , its maximum value, as the weight of the control organs do to the maximum weight attained. The lower 'tail' is therefore cut off more and more as the proportion of C to L increases, so that when C/L is large, the S-shaped effect is lost over the region used as a dose/response curve.

THE GENERALITY OF THE LOGISTIC FUNCTION

The data discussed above, covering a variety of material, have served to indicate that the logistic curve is of wide practical application to material of the type under consideration. A random selection of similar dose/response data from the literature also shows that, whether the authors have given consideration to the mathematical expression of their results or not, few have produced data which do not seem to be better fitted by a logistic curve than by any other simple curve. We have not examined these curves in detail, since we are in a better position to deal with the errors and implications of our own results than with those of other workers, but reference may be made to the papers of Deanesly [1935], Fevold, Hisaw & Greep [1937], Levin & Tyndale [1937], Hamburger & Pedersen-Bjergaard [1937, 1938], Heller, Lawson & Severinghaus [1938], Cartland & Nelson [1938] and Tyndale, Levin & Smith [1938] for curves showing the response of the ovary or uterus of rodents to various gonadotrophins, all suggestive of a logistic rather than of a logarithmic dose response relationship. There are few curves available for thyrotrophic and lactogenic preparations, and of them, those of Smelser [1938] for the response of the chick or guinea-pig thyroid gland do not seem to be fitted

by logistic curves, while the curves of Bates [1937] and possibly those of Lyons [1937] are better fitted by a logistic than by a logarithmic function.

It is not easy to generalize farther in regard to the application of the logistic curve. It may be applicable, and probably is, to a much wider range of data than those we have considered. Thus, the results of Marks [cf. 1936] dealing with the response of the blood-sugar of rabbits to insulin,

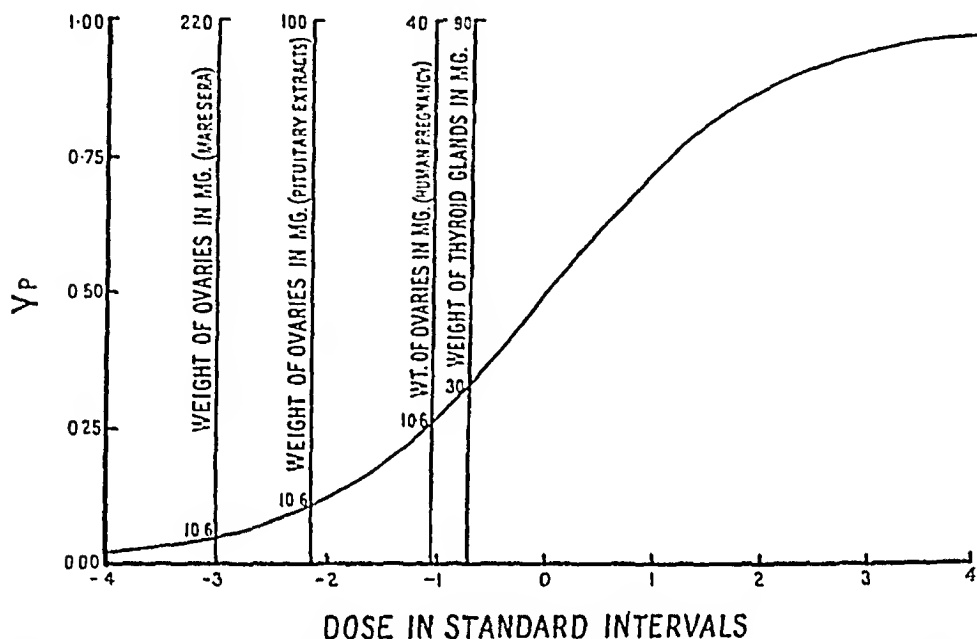


FIG. 8. General logistic as in Fig. 1, showing the point at which the y axis cuts the curve in the assay of various preparations. This is such that y_p at that point = control weight divided by maximum limiting weight, as is indicated by the scales shown on the axes.

of Deane & Parkes [1936] dealing with the response of the prostate and seminal vesicles of the male rat to androgens, of Emmens [1939] dealing with the response of the baby chick's comb to androgens, and of many others who have fitted a logarithmic line to their data, deviate from such a line in the directions to be expected if the true relationship of dose to response were of a logistic nature. It seems probable, therefore, that a great number of dose/response curves are in fact logistic in type, in particular when the response is the weight or volume of an organ or tissue, or a simple function of it.

THE FACTORS WHICH SHAPE THE LOGISTIC CURVE

Yule [1925] discusses in some detail the underlying phenomena which may be supposed to control the growth of populations and Robertson [1908 *a*, *b* and other papers] those which control organic growth, and which give rise in both cases to logistic functions. Neither of these dis-

cussions may, on the face of things, appear to be readily applicable to a discussion of the causes of the logistic nature of a dose/response curve. However, changes correlated with time and with dosage must themselves be correlated in animal tests.

Let us postulate for the moment that the time/response curve of an organ under a constant stimulation, such as that approximately given by a constant daily dose of a preparation, is logistic, having the equation

$$y = \frac{L}{1 + e^{(\beta - t)/\alpha}},$$

when one unit is given per time interval. Let a given total dose produce the same value of y independently of the value of t (i.e. the standard interval is inversely proportional to the daily dose of an extract). Then, if x units are given per time interval, the growth at time $T = t/x$ will be the same as it was in the first case at time t .

Thus

$$y = \frac{L}{1 + e^{(\beta - t)/\alpha}} = \frac{L}{1 + e^{(\beta - Tx)/\alpha}}.$$

Keeping T constant and varying x , the daily dose, we have a second logistic curve relating dose to effect.

With these considerations in mind, a constant daily dose of 2 mg. of the international standard preparation of the serum of pregnant mares was injected into rats, groups of which were killed after the 2nd, 4th, 6th, &c. injection (Table VIII). The time/response curve so obtained is fitted by the logistic

$$y = \frac{220}{1 + e^{(1.35 - t)/1.41}},$$

where t = time of killing, in days, from the first injection (Fig. 9). With this curve, the control ovarian weight is taken as 9.6 mg. instead of 10.6 mg., giving a value of $B = 3.08$, as the body-weight of the controls was less than that usually taken (Table VIII). This follows since the controls were killed at the beginning of the test, not at the end of the usual 5-day period. With the exception of the lowest total dose given (4 mg.), each total dose has elicited a rather higher response than the same total dose in the 5-day assay curve for the standard preparation. It is probable that the higher responses obtained in this series of tests are due either to the use of a different batch of rats or to the fact that these particular tests were conducted by myself, instead of, as is usual, Dr. I. W. Rowlands. The effect of a given total dose is, however, thus seen to be approximately constant and to be little dependent, within the limits 2-10 days, and 4-20 mg. respectively, on the time during which it is administered. It would appear, therefore, that the response to this extract of the serum of pregnant mares follows the time relationship expressed by equation (1) within the limits

necessary for the investigation of the dose/response curve. With this gonadotrophin, the logistic nature of the dose/response curve may be assumed to follow directly from the logistic time/response curves which

Table VIII. *Data relating to the response of the ovary of the immature female rat to a constant daily dose of 2 mg. of the international standard preparation of the serum of pregnant mares*

Injection period (days)	Total dose (mg.)	No. of rats	Average body-weight at killing (g.)	Average weight of ovaries (mg.)	No. of mg. equivalent to a standard interval
0	0	22	40	9.6	2.82
2	4	8	41	25	
4	8	8	50	79	
6	12	9	54	188	
8	16	9	54	216	
10	20	8	60	224	

various constant daily amounts should give. It is improbable that very low doses would obey this time/response law, and as the ovary is limited in its capacity to respond within a short period, high doses do not elicit

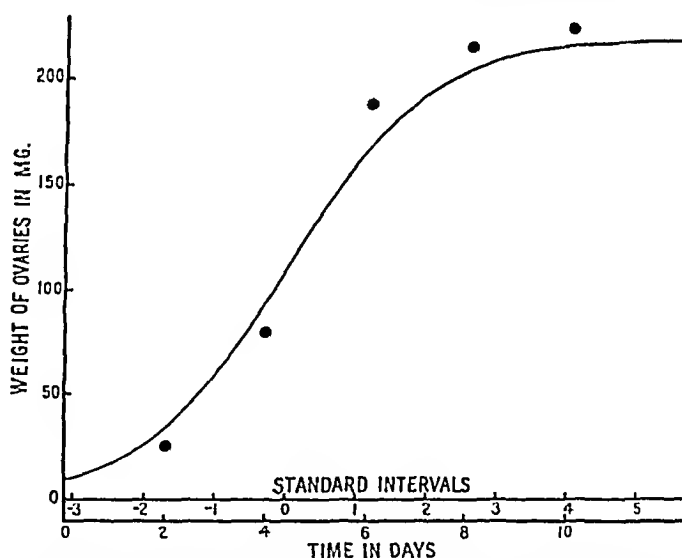


FIG. 9. The logistic curve fitted to the data for the response of the ovary of the immature rat to the international standard extract of the serum of pregnant mares (Table VIII) when a constant daily dose of 2 mg. was injected.

a maximal response in 2 or 3 days. Over the time range of 5 days, however, no significant departure is apparent.

Deanesly's [1935] data dealing with the effect of continued injections of a constant daily dose of an extract of horse pituitary glands (AP2B) or of human pregnancy urine (UP10) are in general agreement with our

findings with the extract of pregnant mares' serum. After 20 or more days of injection of AP2B, however, the ovarian weight decreases, probably due to the formation of antihormones in the serum of the test animal. When a given total dose of either extract was injected during different periods of time, the response did not vary greatly, but was regularly lower when only 3 days were allowed for ovarian growth than when the test period was 5 or 10 days. It may be assumed, therefore, that the response of the rat's ovary to these gonadotrophins also tends to follow a logistic time/response curve when a constant daily dose is administered, but that the limits between which the law holds are not wide.

Rowlands & Parkes [1934] examined the corresponding relationships for thyrotrophin. They also found that the influence on the response of the period over which a given total dose is injected is slight, when periods of 2-10 days are considered. However, when a constant daily dose was injected the maximum weight of thyroid glands attainable was found to fall as the daily dose was decreased from 5 to 2 mg. of T17B. The response to a daily dose of 5 mg. given for periods of 2-20 days is fitted well by the logistic

$$y = \frac{85}{1 + e^{(2.15 - t)/3.53}},$$

but that to a daily dose of 2 mg. has an upper limit of 60, and is approximately fitted by the logistic

$$y = \frac{60}{1 + e^{(-t)/2.53}},$$

the point of inflexion corresponding with the origin. Here, the lower limiting response is compensated by a much smaller standard interval than would be expected on the assumption that the standard interval is proportional to the daily dose. It is still possible, however, that in the earlier stages of the response to 2 mg. per day the curve follows a law similar to that found to fit the data for the gonadotrophins, and that a 'falling off' in response begins after a short period.

Injecting a constant total amount of prolactin (AP8A and AP8B) Rowlands [1937] found that a definite maximum occurs in the response when a 6-day test period is chosen. With a longer or a shorter interval, the response falls rapidly. The time/response curve for a constant daily dose of prolactin was not investigated, but it clearly cannot bear a similar relation to the daily dose as that postulated in the case of extracts of pregnant mare serum.

We see, therefore, that, however attractive the idea that the dose/response curve is governed by logistic time/response curves with standard intervals inversely proportional to the daily dose may be, its application is limited. It appears to be an adequate description of what occurs in the

case of some gonadotrophins, but to break down when thyrotrophin or prolactin is considered.

The limiting weight of the rat ovary under different types of stimulation affords considerable scope for discussion. The action of extracts of pregnancy urine on the hypophysectomized rat shows that it does not contain follicle-stimulating substance (FSH) [cf. Selye, Collip & Thomson, 1933; Leonard & Smith, 1934]. The low maximum which is attainable in the ovarian weight of rats injected with such extracts is a consequence of this, as any follicular stimulation which may occur prior to luteinization depends on the rat's own pituitary gland. With extracts of pituitary origin, containing various amounts of both FSH and LH (luteinizing hormone), higher ovarian weights may be obtained, but the co-operation of the pituitary gland of the test animal again seems necessary for the production of maximal effects. Noble, Rowlands, Warwick & Williams [1939] have recently compared the effects of different gonadotrophic extracts in normal and hypophysectomized rats, and further references to earlier work may be obtained from their paper. They conclude that the primary action of the injected gonadotrophin on the ovary probably leads to a secondary stimulation of the pituitary gland, which secretes additional amounts of hormone. Whatever may be the mechanism, it is quite clear that no gonadotrophic extract yet investigated can be confidently regarded as producing effects on the ovary, in the type of test under consideration, which are uncomplicated by the additional action of endogenous gonadotrophin. However, the maximum ovarian weight attainable is found to depend on the proportion of FSH to LH which is present in the original extract, the more FSH present, the higher the maximum ovarian weight [cf. Deanesly, 1939]. Thus, extracts of the serum of pregnant mares, which contain very little LH, will produce the highest ovarian weights found in gonadotrophic tests with rats.

There is a further peculiarity of extracts of pregnant mares' serum, in that the active principles are not as rapidly eliminated after injection as are gonadotrophins from other sources [Cole & Hart, 1930]. A single injection of 5 or 7 mg. of PMS14, for instance, produced after a period of 5 days ovarian weights which equalled those produced by injecting the same total amount in five daily doses [Rowlands & Williams, 1941]. If the animals receiving a single initial injection are killed on the 3rd day, however, the ovaries have not yet reached the weight seen at the end of 5 days, showing that active material is still available at that time. This occurs in both normal and hypophysectomized animals. The greater part of the growth following such an injection occurs, however, during the first 3 or 4 days, as is also seen by comparing the results shown in Table VII with those at the top of Table II, the time during which a given total

amount is injected having little effect on the response. This property of mare serum extracts, therefore, has little effect on the consideration of dose/response curves except that relatively high doses cannot be expected to take full effect in a very short period of time.

The small difference found between the maximum crop-gland weights produced by different preparations of prolactin do not seem to be related to any other differences between the extracts concerned. Most of these were prepared by similar methods from ox pituitary glands, and furthermore, plurality of types of prolactin has not been claimed.

Extrapolation of the logistic dose/response curves we have dealt with is not without meaning. It presents no difficulties for positive values of x , the dose. Dr. I. W. Rowlands (personal communication) has been unable to demonstrate any further increase in the response of rat ovaries to an extract of urine of pregnancy when given in 30 times the dose sufficient to cause a maximal response. As was indicated on p. 205, the curve may still mean something when negative dosages are considered, as it may be taken to represent the growth of the ovary under a lower level of stimulation than is normally provided by the rat's own pituitary gland. Naturally, there is a limit to extrapolation in a direction of negative dosage, but the curve has the virtue of never showing a negative ovarian weight, but rapidly approaches zero as gonadotrophin is imagined to be withdrawn from the circulation. In the hypophysectomized rat, the ovary regresses to a weight of about 5 mg., but a lower ovarian weight would doubtless be seen in animals deprived of their pituitary gland at birth or at an earlier stage than that at which the operation is normally performed, and in which the complication of regression from a higher to a lower weight, with a certain amount of indestructible tissue involved, would not occur. Chou & Liu's [1937-8] data were fitted by logistics which were approximately asymptotic to the control ovarian weight, but I have been unable to support this finding.

CONVERSION OF A LOGISTIC TO A LINEAR FUNCTION

When dealing with quantal ('all or none') responses a function commonly used is the 'normal equivalent deviation' [cf. Gaddum, 1933]. It is the value of the deviation from the mean of a normal curve which is equivalent to any given percentage, and is measured with the standard deviation as unity. By the use of this quantity, a linear regression function can often be obtained.

By analogy with the normal equivalent deviation, we may use the 'equivalent standard interval' when dealing with logistic curves. This quantity, measured with the standard interval as unity, is equivalent to X_c of the foregoing discussions. The value of X_c , however, is only known

when the data have already been fitted by a logistic curve, hence it is best to rename the quantity, which we shall call Y . Y is therefore such that

$$y_p = \frac{1}{1 + e^{-Y}},$$

or

$$Y = -\log_e \left(\frac{1}{y_p} - 1 \right),$$

and its values may be read off from Yule's [1925] tables of the ordinates of the logistic, equating Y to τ in those tables. When y_p is 0.5, $Y = 0$; for values of y_p above 0.5, Y is positive; for values of y_p below 0.5, it is negative.

If Y is now plotted against the dose, the data fall on a straight line if they are fitted by a logistic function, experimental error excepted. This has been done for the data in Table II and Fig. 2, and Fig. 10 shows the result. Points falling on or above the limit, L , cannot be included in such a figure, but their contribution to the determination of the dose/response line is supposed to have been made in finding a value for L . In Fig. 10 both axes have the same scale, measured in standard intervals, since the number of mg. of each preparation equivalent to a standard interval was already known.

Y is to be used when fitting a line to experimentally determined points, as can be done when the data resemble those under discussion, where L is known, and it may be plotted graphically against the dose, or the line may be fitted by the method of least squares. The weight factors to be included in the calculations would depend on the values taken by σ_Y .

Since

$$\begin{aligned} \frac{1}{y_p} \frac{dy_p}{dY} &= 1 - y_p, \\ \frac{dY}{dy_p} &= \frac{1}{y_p(1-y_p)}. \end{aligned}$$

But

$$\begin{aligned} \sigma_Y &= \sigma_{y_p} \frac{dY}{dy_p} \\ &= \frac{\sigma_{y_p}}{y_p(1-y_p)}, \end{aligned}$$

where σ_{y_p} is determined experimentally, and equals σ_p/L . The weight factor, w' , corresponding with a given average response, \bar{y}' , would then be given by

$$\begin{aligned} w' &= \left\{ \frac{y'_p(1-y'_p)}{\sigma_{y'_p}} \right\}^2 \\ &= \left\{ \frac{\bar{y}'(L-\bar{y}')}{L\sigma_{y'}} \right\}^2 n', \end{aligned}$$

where $n' =$ the number of animals in the group.

If Y is plotted against x and the points fitted by

$$\eta = \frac{\beta - x}{\alpha} = a - bx$$

by the method of least squares, and a wrong value of L has been used, then the scatter of points about the line will be greater than if the right value of L had been used. The error of the determination of L will thus be taken into account automatically in calculating the error of a and b .

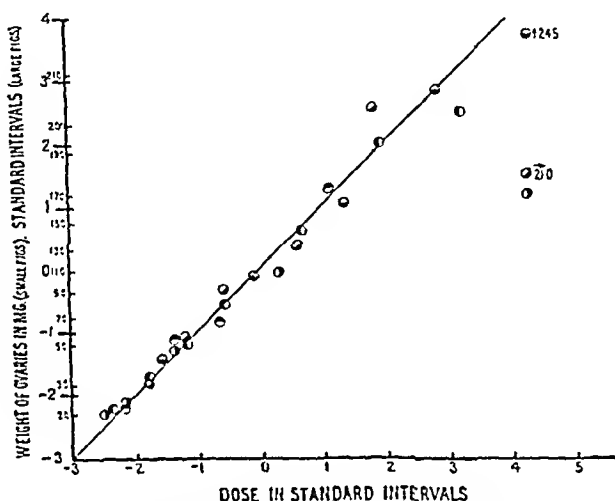


FIG. 10. The logistic curve of Fig. 2 expressed as a straight line, by adjustment of the ordinates (see text).

If the variance of Y is independent of x , within the limits of random sampling, a test for linearity of regression will be a test of goodness of fit. Otherwise, when weights have been taken inversely proportional to the variances of Y , we may calculate

$$\chi^2 = \frac{S\{w(\bar{Y} - \eta)^2\}}{k-2},$$

where \bar{Y} is the mean value of Y , and k is the number of doses and enter the table of χ^2 with $k-2$ degrees of freedom. This method seems the best way of fitting, when the function is to be used for assay purposes, and the error of a is important.

A further method of fitting a logistic curve to the data is therefore available, by which the plotting of a curve is avoided when the results are expressed graphically. The method has also the great advantage that the errors involved are calculable, and that a technique of assay may be based upon it.

SUMMARY

1. An examination of the response of the ovary of the immature rat to various gonadotrophins, of the thyroid gland of the guinea-pig to thyrotrophin, and of the crop-gland of the pigeon to prolactin, has shown that, as might be expected, the statistically convenient log-dose/response relationship is tenable only over a limited range. This range is sometimes smaller than is desirable in practice.

2. The results are, instead, fitted very well by logistic curves. The general logistic curve is such that

$$y = \frac{L}{1 + e^{(\beta - x)/\alpha}},$$

where y is the response, x the dose, L the limiting value of y and α and β are constants. The curve is so fitted that the y -axis cuts the curve at a point C , which is equal to the weight of the glands of unstimulated controls. For a given value of L , this procedure fixes the value of β/α .

3. Extracts fitted by curves with the same value of C and L may be grouped on to a single curve. The number of mg. of each extract equivalent to one unit of α , the standard interval, is then inversely proportional to the potency of the extract. By taking α as unity, the equation to the logistic curve becomes:

$$y = \frac{L}{1 + e^{B - X}},$$

where $B = \beta/\alpha$ and $X = x/\alpha$, the dose being measured in standard intervals.

4. Further properties of the logistic curve and methods of fitting it to data are discussed. The curve may be converted to a straight line by the use of the 'equivalent standard interval', analogous with the normal equivalent deviation used in dealing with quantal responses. The error of an estimate of relative potency may also be determined when the latter method of fitting is used.

5. The logistic nature of the dose/response curves may be explained in some cases by the growth of the test-object under constant stimulation being itself logistic—i.e. exhibiting a logistic time/response relationship. This appears to be true for some gonadotrophic extracts, notably those of the serum of pregnant mares, but does not hold throughout.

6. It is suggested that the logistic function may be of application in other quantitative assays, many of which appear to possess dose/response relationships which give curves closer to a logistic than to a logarithmic type. Its usefulness for assaying will depend on the statistical method which may be evolved for use with the curve, but as a description of the dose/response relationship, it is clearly superior to the form $y = a + b \log x$.

The great majority of the tests discussed in this paper were carried out

by Dr. I. W. Rowlands. Some of his results have already been published in the various works to which reference has been made, but a large number have not previously appeared in print. I am, therefore, deeply indebted to Dr. Rowlands for permission to make such extensive use of them. I am also very grateful to Prof. M. Greenwood, F.R.S., and Dr. J. O. Irwin for their criticism and helpful suggestions.

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FURTHER EXPERIMENTS ON THE CONTINUED TREATMENT OF LACTATING COWS WITH ANTERIOR PITUITARY EXTRACTS

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EARLIER experiments have shown that when repeated injections of a prolactin preparation are given to cows in declining lactation, a substantial stimulation of milk production occurs, though later this stimulus declines and finally vanishes, despite continued treatment [Folley & Young, 1939]. Further experiments have now been carried out to determine if the initial response would disappear with continued treatment with other pituitary preparations, and if the development of such resistance to the action of the extract was associated with the appearance, in the blood of the treated cows, of anti-prolactin [Young, 1938 *c*; Bischoff & Lyons, 1939] activity.

Folley & Young [1938] found that the stimulating influence on milk production of cows in declining lactation of a *single* injection of various anterior pituitary fractions was more closely correlated with their glycotropic (anti-insulin) activities than with their prolactin¹ content. It therefore seemed desirable to investigate the influence on milk production of continued injections of a pituitary preparation [Young, 1938 *b*] having glycotropic activity but no detectable prolactin content.

METHODS

Preparation of extracts

Three types of anterior pituitary extract were used in this investigation. All were prepared from absolutely fresh ox glands brought from the slaughter-house in solid carbon dioxide snow [cf. Young, 1938 *a*]. pH determinations were made colorimetrically as before described.

Crude alkaline extract was prepared as previously described [Young, 1938 *a*] by grinding the dissected frozen anterior lobes with saline at pH 8–8.5 in the cold room. The crude extract, obtained by spinning off the residue, was further clarified by high-speed Sharples centrifugalization,

¹ In this paper the term prolactin will be used to denote the anterior pituitary substance which induces hypertrophy of the pigeon crop-gland, without reference to the question of whether or not such a substance is, *per se*, lactogenic in mammals.

such treatment removing suspended matter responsible for the unpleasant local reactions previously observed when single injections of a crude anterior pituitary extract were given to cows. In the present investigations no local reactions of any kind were observed to follow prolonged treatment with the pituitary extracts we have used.

A solution of inert protein material, for control injections, was prepared from calf thymus gland in a manner similar to that for the crude alkaline extract of anterior pituitary tissue. The cows treated with this extract developed moderate sized lumps at the site of injection half-way through the experiment, but these had entirely disappeared two days later.

The final concentration of both the crude pituitary extract and the thymus extract was such that 4 ml. contained the material extracted from 1 g. of fresh tissue.

The *prolactin preparation* consisted of the fraction of the crude alkaline extract soluble at pH 8 but insoluble at pH 5 [Young, 1938 *a*]. This was dried by precipitation with excess alcohol at pH 5, the solid material obtained representing 1.4 g. per 100 g. of fresh anterior lobe tissue.

A *glycotropic preparation* was prepared by heating crude alkaline extract at pH 10 on a boiling water bath for one hour [cf. Young, 1938 *b*]; after cooling, the solution was adjusted to pH 8 and the insoluble material spun off. 4 ml. of this solution were taken to be equivalent to 1 g. of fresh ox anterior lobe tissue.

Biological assays

The biological activities of the extracts used are summarized in Table I.

Prolactin was assayed by the systemic method of Rowlands [1937]. The results are expressed approximately in terms of International Units.¹

Table I. *Biological activities of the extracts used*

Anterior pituitary extract	Prolactin activity (Approx. I.U.)	Thyrotropic activity (Approx. I.U.)	Glycotropic activity (units)
Crude alkaline preparation	18.4/ml.	2.8/ml.	1.5/ml.
Glycotropic preparation	very small*	very small	0.5/ml.
Prolactin preparation	5.1/mg.	20/g.

* No response when 2 ml. was injected daily for six days into each pigeon.

Thyrotropic activity was determined on immature guinea-pigs by the method of Rowlands & Parkes [1934]. The results are expressed approximately in terms of International Units.²

Glycotropic activity was determined by the method of Young [1938 *a*]. This consists in determining the minimum amount of extract, administered

¹ Cf. Bulletin of the Health Organization of the League of Nations [1928], 7, 817.

in two doses, required to neutralize the hypoglycaemic action of 2 units of insulin in the fasting rabbit.

Experimental animals

The cows were of the Ayrshire and British Friesian breeds belonging to the Agricultural Research Council Field Station, Compton, Berks. Four groups of four animals were used, each consisting of three Ayrshires and one British Friesian. Each group was so constituted that, as far as possible, its members were comparable as regards milk yield, etc., with those in the other three groups. Data relating to the experimental animals are given in Table II.

Table II. *Data relating to the cows used*

Cow no.	Breed	Group	Extract injected	Date of last calving	Days pregnant at time of first injection
1	British Friesian	A	Thymus	15/12/39	21
2	Ayrshire	A		22/10/39	20
3	"	A		28/10/39	—
4	"	A		1/10/39	—
5	Ayrshire	B	Glycotropic preparation	25/10/39	—
6	"	B		22/9/39	4
7	British Friesian	B		10/10/39	—
8	Ayrshire	B		11/9/39	121
9	British Friesian	C	Crude alkaline extract	12/9/39	92
10	Ayrshire	C		23/9/39	—
11	"	C		2/10/39	121
12	"	C		7/8/39	112
13	Ayrshire	D	Prolactin	19/10/39	111
14	British Friesian	D		8/10/39	54
15	Ayrshire	D		21/10/39	89
16	"	D		7/10/39	—

Injection of extracts

The injections of extract were given subcutaneously on alternate days during the afternoon milking period, each cow receiving altogether eleven injections spread over 22 days.

Group A, the control group, received inert protein in the form of 10 ml. of thymus extract for each cow on every second day. The cows in group C received similar injections of 10 ml. of the crude alkaline pituitary extract, equivalent to 2.5 g. of fresh tissue, while those in group D were given injections of 250 mg. of the prolactin preparation, in 10 ml. of solution. The animals in group B received seven injections each of 10 ml. of the glycotropic preparation, followed by four of 20 ml.

The period of injections was preceded by a control period of 12 days, during the greater part of which the milk yields of all the cows were recorded.

Analysis of milk

For analysis, milk samples were taken three times weekly at the morning and evening milking throughout the control and experimental periods, and for each group, samples of the morning and evening yields were combined in the ratio of the total yields, to give a composite group sample. Percentages of fat and of non-fatty solids were determined, as previously described [Folley & Young, 1938].

RESULTS

Milk yield

Average values, for three-day periods, of the aggregate milk yields for each group are plotted in Fig. 1. In each instance the best straight line for the pre-injection data was determined by the method of least squares, and projected so as to cover both the injection period and subsequently (Fig. 1). This represented the milk yield expected if treatment with extract had not been instituted [cf. Folley & Young, 1938].

From Fig. 1 it will be seen that the milk yield of the cows receiving the crude pituitary extract rose during the first half of the period of injections, and then slowly declined at about the rate obtaining during the pre-injection period, despite the continued treatment. The cessation of treatment after a period of 22 days did not appear to affect the rate at which the milk yield subsequently declined. The maximum aggregate milk yield during the injection period was 87 lb./day or 24% above that expected if treatment had not been instituted. The average amount of milk secreted daily in excess of that expected, during the period of injections, was 11.5 lb. or 16.7% above the expected total daily yield for the four cows.

Treatment with the prolactin preparation also resulted in an initial stimulus to milk production (Fig. 1) but, despite maintenance of treatment, the yield slowly fell to that expected in the absence of injections, reaching this value by the fifteenth day of treatment. The average excess daily milk yield during the injection period was 3.8 lb., or 5.2% above that expected.

The injections of the glycotropic preparation appeared, on the whole, to exert no obvious influence on milk production (Fig. 1), and, although there may have been a very slight initial stimulus, this was not maintained, even though the amount of extract injected was doubled on the twelfth day of treatment. The average daily milk yield during the period of injections was similar to that to be expected without treatment.

Constituents of the milk

In no instance did the injections induce any significant alteration in the proportion of fatty or non-fatty solids in the milk (Table III). This

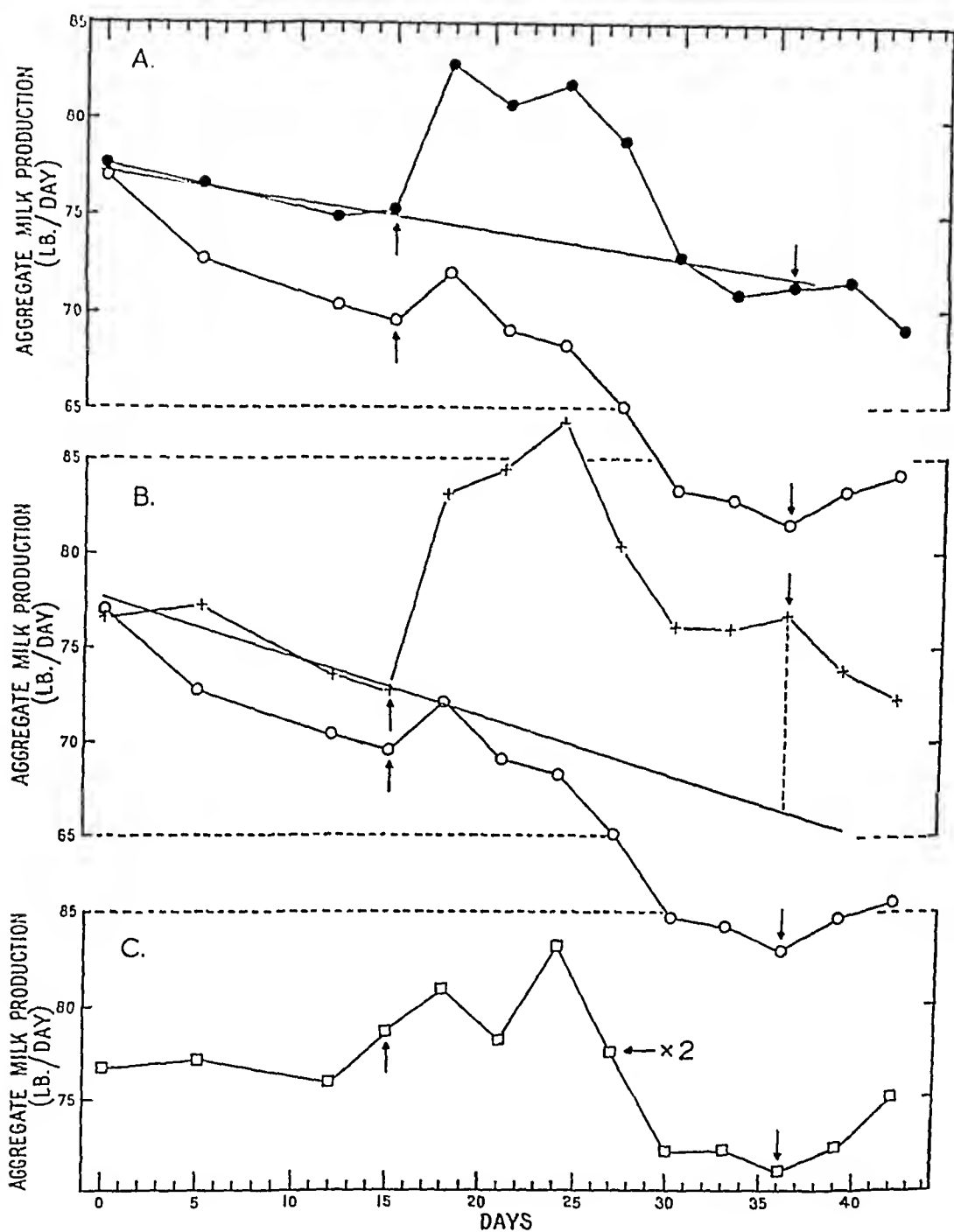


FIG. 1. The influence on milk production in groups of four lactating cows of treatment with anterior pituitary extracts.

Injections of extract were given every second day from the 16th to 36th day (inclusive) of the experiment.

- ↑ ↓ Signifies the last observation before injections began.
 ↓ Signifies the last observation before injections ceased.
- (A) ●—● Treatment with 250 mg. (= 1275 I.U.) of prolactin.
 ○—○ Control treatment with thymus extract.
- (B) +—+ Treatment with 10 cc. of crude extract containing 184 I.U. of prolactin.
 ○—○ Control curve.
- (C) □—□ Treatment with a glycotropic preparation.
 ← × 2 Signifies that the amount of extract injected was doubled from that point onwards.

result is in contrast to that previously obtained [Folley & Young, 1939] in experiments in which repeated injections of a prolactin preparation resulted in a substantial increase in milk fat content.

Table III. *Data relating to solids content of milk*

	Average % of fat in milk				Average % of non-fatty solids in milk			
	During pre-injection period	During first half of injection period	During second half of injection period	After injection period	During pre-injection period	During first half of injection period	During second half of injection period	After injection period
Extract injected								
Thymus	3.62	3.71	3.71	3.52	8.30	8.24	8.04	8.00
Crude anterior pituitary preparation	3.79	3.84	3.79	3.61	8.22	8.27	8.45	8.33
Prolactin	3.57	3.59	3.60	3.35	8.09	7.99	8.01	8.07
Glycotropic preparation	3.88	3.85	3.97	3.67	8.39	8.43	8.39	8.33

Tests for anti-prolactin activity in the blood

As the subsidence of the stimulating influence, on milk production, of treatment with the prolactin preparation (Fig. 1) might be due to the appearance of anti-prolactin activity in the blood of the treated cows, 20 ml. of blood were drawn from the jugular vein of each animal two days after the last injection of pituitary extract. Blood was thus obtained not only from the cows which had received treatment with the prolactin preparation but also from those which had been treated with the crude pituitary extract or with the thymus extract, and from those receiving the glycotropic preparation.

For each group a pooled sample was constituted from equal volumes of serum from each of the four cows, and tested for anti-prolactin activity by the method of Young [1938 c]. Groups of six pigeons were used in these tests and the inhibitory effect of injecting 1 ml. of serum daily on the crop-stimulating action of a preparation of prolactin obtained by the method of Bates & Riddle [1935] was determined. The group of pigeons receiving serum from the cows which had been treated with the thymus extract served as controls. The results, which are given in Table IV, provide no evidence of the presence of anti-prolactin activity in the serum from any of the groups of cows treated with pituitary extract. They suggest, in fact, that some unmasked prolactin was present in the serum.

Summary of results relating to the influence on milk-yield of a single injection of pituitary extract

The evidence, on the basis of which it was assumed that prolactin was not the only pituitary substance concerned in the stimulation of milk production by the lactating cow [Folley & Young, 1938], was drawn from experiments in which the effect of a single injection of pituitary extract was studied. As in the present and in a previous investigation [Folley &

Young, 1939] injections of extracts were given at intervals of two days, it was possible to determine the effect of the first injection alone on the

Table IV. *Test for presence of anti-prolactin in blood sera of cows*

Group of cows from which pooled serum was prepared	Extract injected into cows	No. of pigeons in group used for test	Total dose of prolactin given (Approx. I.U.)	Total amount of serum injected in six daily portions into each pigeon (ml.)	Mean crop-weight as % body-weight
A	Thymus	6	13.5	6	1.22
B	Glycotropic preparation	6	13.5	6	1.33
C	Crude extract	6	13.5	6	1.22
D	Prolactin	6	13.5	6	1.28
—	—	40	13.5	0	1.10

average milk production during the following two days. Table V summarizes all the results thus far obtained in the present and two previous investigations [Folley & Young, 1938, 1939] on the influence of a single

Table V. *Summary of the results of experiments on the influence of a single injection of pituitary extract on milk yield*

Type	Extract		No. of cows in group	Average daily yield over two days before injection	Average % increase in milk-yield over two days after injection	Reference*
	Amount injected	Prolactin content (I.U. Approx. values)				
Crude alkaline extract	2.5 g.-equivalent	181	4	18.1	9.4	(c)
	5.0 "	300	6	25.9	9.8	(a)
	10.0 "	600	6	23.0	11.8	(a)
Prolactin from fresh tissue	10.0 "	330	3	16.7	8.5	(a)
	10.0 "	330	4	32.2	11.1	(b)
	250 mg.	1275	4	18.7	9.7	(c)
Prolactin from commercial dried gland	200 mg.	800	3	23.3	0.3	(a)
	750 mg.	2500	2	23.2	3.8†	(a)
Thyrotropic preparation	10 g.-equivalent	Very small	3	19.3	8.5	(a)
	10 "	" "	3	22.7	6.7	(a)
	10 "	" "	3	22.2	4.1	(a)
	(+posterior lobe extract)					
	10 g.-equivalent	" "	4	28.7	5.9	(b)
Thyrotropic preparation from commercial dried gland	250 mg.	" "	3	20.1	0.0	(a)
	500 mg.	" "	3	20.7	1.8	(a)
Glycotropic preparation	2.5-5.0 g.-equivalent	Very small	4	20.0	0.6	(c)
<i>Control</i>						
i. Liver	5 g.-equivalent	0	6	26.0	0.2	(a)
	10 "	0	6	21.6	-2.7	(a)
	10 "	0	4	28.1	0.1	(b)
ii. Thymus	2.5 "	0	4	17.4	2.7	(c)

* References (a) = Folley & Young [1938]; (b) = Folley & Young [1939]; (c) = Present investigation.

† Prolactin given on second day also.

injection of pituitary extract on milk production in the lactating cow during the two days following the injection, and illustrates the lack of relationship between the prolactin content of the different pituitary preparations and their influence on milk production in these experiments. Furthermore, no precise correlation is found to exist between glycotropic activity and influence on milk production, and it seems unlikely that there is a single specific substance concerned.

DISCUSSION

The results of the present investigation have confirmed the impossibility of maintaining the initial stimulus to milk production when cows in declining lactation receive regular injections of prolactin over a period of two or three weeks. The influence of injections of a crude alkaline extract was also not maintained, although, in this experiment, the milk production did not fall during the period of injections to the value expected if treatment with extract had not been instituted, as was the case with the prolactin preparation (Fig. 1). In neither group of cows was evidence found for the development of anti-prolactin activity in the blood, so that the diminution in response cannot be ascribed to the development of large amounts of substance inhibiting the action of prolactin (antihormone). The possibility that the stimulus subsides because the supply of suitable metabolites for conversion to milk becomes diminished cannot be ruled out, although the constancy of the mean contents of fatty and non-fatty solids in the milk before and during the period of injections (Table III) lends no support to this view.

In the present investigation the dose of prolactin injected daily was obtained from about 30 g. of fresh anterior lobe, whereas the dose of prolactin previously used [Folley & Young, 1939] was equivalent to only 10 g. of fresh tissue. The prolactin used in the present investigation had been dried and stored for some time, a process which results in loss of glycotropic activity, whereas the previous 'prolactin-C' was stored in solution and used within a few days of preparation. It is not clear why the milk-fat content in the present experiments remained constant when the cows were treated with crude extract or with prolactin, whereas a substantial increase was previously found to follow prolonged injections of a somewhat different preparation of prolactin; the higher glycotropic activity of the dose of extract used in the previous experiments may be responsible.

As in our previous work on the effect on milk production in cows of a single injection of pituitary extract [Folley & Young, 1938] we have again, in the present investigation on the influence of prolonged treatment, found a poor correlation between the prolactin content of our pituitary extracts

and their ability to stimulate milk production in a cow in declining lactation. Thus the data in Table I show that the daily dose of prolactin was nearly seven times as active in stimulating the pigeon crop-gland as was the daily dose of crude extract, yet the latter was over three times as active in stimulating milk production. We have previously discussed the question of the identity of the 'lactogenic hormone' of the anterior pituitary lobe and concluded that there is no reason to believe in the existence of a single pituitary hormone stimulating lactation [Folley & Young, 1938]. In a recent paper Bergman, Meites & Turner [1940] suggest that the term 'lactogenic' should be applied only to an anterior lobe hormone which *initiates* lactation, and state their belief that the pigeon-crop stimulating factor (prolactin, galactin, mammotropin) is identical with the lactogenic factor. Evans [1939] has also come to a similar conclusion. It is, however, already admitted that purified prolactin is incapable of initiating lactation in hypophysectomized animals [Nelson & Gaunt, 1936; Gomez & Turner, 1936] unless adrenotropic hormone, and sometimes glucose, is administered as well [Gomez & Turner, 1937; Nelson & Gaunt, 1937]. On the basis of experiments with hypophysectomized animals, therefore, it is legitimate to argue either that prolactin is not a specific lactogenic hormone or alternatively that prolactin and adrenotropin have similar claims to the title, since either may initiate lactation in hypophysectomized animals in the presence of the other. Bergman *et al.* [1940] consider that the term 'lactogenic' should not be applied to a pituitary substance which merely increases the milk yield of an animal already lactating. If it is desirable to draw such a distinction, then we suggest that the term 'galactopoietic' be applied to an anterior pituitary substance which increases the yield of an animal already producing milk. The results summarized in Table V show that preparations of prolactin which are low in glycotropic activity are also low in galactopoietic power, although, if crop-stimulating activity is a true index of lactogenic ability, such extracts should be highly effective in initiating lactation in hypophysectomized animals despite their low activity in stimulating an already existing lactation.

There appears to us no reason why a woman whose milk supply is deficient and who is about to receive treatment with a pituitary preparation, should be considered more closely analogous to the hypophysectomized guinea-pig or the intact pigeon, than to the cow in declining lactation. It therefore seems to us that an investigation of the galactopoietic activities of pituitary preparations is at least as important, from the clinical aspect, as the determination of their lactogenic power, using the terms lactogenic and galactopoietic in the sense defined in the present paper.

Prolactin preparations low in glycotropic activity are also low in galacto-

poietic power [Folley & Young, 1938], while in certain instances prolactin is incapable of initiating lactation in hypophysectomized animals unless adrenotropin is also administered [Gomez & Turner, 1937; Nelson & Gaunt, 1937]. Now Jensen & Grattan [1940] claim that the glycotropic substance and adrenotropin are identical. Without offering any opinion as to the validity or otherwise of this claim we would point out that if its truth were established the failure of purified preparations of prolactin to exhibit either lactogenic or galactopoietic activities in certain circumstances might be correlated.

We must emphasize that although previously we observed a relationship between the glycotropic and galactopoietic activities of anterior pituitary preparations [Folley & Young, 1938] such a simple correlation is not borne out by the present findings, in that the glycotropic preparation with little or no prolactin content had no obvious galactopoietic effect (Fig. 1). It is therefore possible that the presence of both prolactin and the glycotropic substance are necessary for a preparation to possess galactopoietic activity, as, indeed, both adrenotropin and prolactin are required for the initiation of lactation in the hypophysectomized guinea-pig. If this is so, the assumption of Jensen & Grattan referred to above would clearly assist in drawing a closer analogy between the requirements, for the stimulation of milk production, of the hypophysectomized guinea-pig and of the lactating cow.

SUMMARY

Repeated injections, spread over 22 days, of a crude anterior pituitary extract into cows in declining lactation, increased the average daily milk production during the period of injections to an average level 16% above that expected in the absence of treatment. Similar injections of a preparation of prolactin, with pigeon crop-gland stimulating activity about seven times that of the crude extract, increased the average milk yield to only 5% above that expected. In both instances the stimulus to milk production declined in magnitude towards the end of the period, but no evidence was found for the presence of anti-prolactin activity in the serum of the cows at this time.

Similar treatment with a pituitary preparation having glycotropic activity but no detectable prolactin had no obvious influence on the milk production of cows in declining lactation.

In no instance was the content of fat or of non-fatty solids in the milk affected by the treatment with pituitary extract.

The significance of these results with respect to a belief in the existence of a single milk-stimulating hormone secreted by the anterior pituitary gland is discussed.

It is a pleasure to record the generous and willing co-operation of Major G. W. Dunkin, M.R.C.V.S., D.V.H., Director, and Mr. S. J. Edwards, M.Sc., M.R.C.V.S., and Mr. E. A. Macmillan, of the Agricultural Research Council's Field Station, Compton, Berks. Our thanks are also due to Dr. G. W. Scott Blair for affording facilities for determination of milk solids.

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CALCIUM AND PHOSPHORUS METABOLISM IN THYROTOXICOSIS

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THE object of the experiments described was to determine whether the decalcification occurring in the human skeleton in thyrotoxicosis can be attributed to thyroxine or to some other factor.

A probable relationship between the thyroid gland and calcium and phosphorus metabolism has been suggested by many workers who have based their evidence on clinical grounds, the radiological study of bones, biochemical analyses on blood-serum, calcium and phosphorus balance experiments, and finally the post-mortem appearance of the skeleton.

EVIDENCE OF DECALCIFICATION IN THYROTOXICOSIS

Clinical evidence

Von Jaksch & Rotky [1908-9] reported softening and bending of the bones in a girl of 20 suffering from hyperthyroidism, and Bernhardt [1927] observed a similar case. These authors suggested that the osteomalacia resulted from a decalcification of the skeleton, and this was attributed to thyrotoxicosis.

X-ray appearance of bones

Kummer [1917] appears to be among the first observers to draw attention to the occurrence of osteoporosis in thyrotoxicosis. In the course of a paper on the high calcium excretion in this disease, he wrote: 'Cette déminéralisation calcaire paraît porter surtout sur le système osseux, à en juger d'après les radiographies des basedowiens.' Phummer & Dunlap [1928] and Aub, Bauer, Heath & Ropes [1929] confirmed that in exophthalmic goitre of longer duration the continued calcium loss was apparent in X-ray pictures of the bones. Golden & Abbott [1933] in a radiographic study of 110 cases of thyrotoxicosis found 78% were normal, 11% showed slight decalcification and 11% showed definite decalcification. In 51 cases of hypothyroidism similarly studied they found 73% were normal, 18% showed slight decalcification and 9% definite decalcification. They concluded that whereas an abnormal elimination of calcium might take place in hyperthyroidism, it was doubtful whether the thyroid had anything to do with the density of the bones. Stettner [1931] has reported an interesting case of decalcification of the bones of a child resulting from

the continued administration of thyroid, and after the thyroid had been discontinued the bones became denser.

Serum calcium studies

Among others, Leichter [1923], Rabinowitch [1924], McCullagh [1928], Wade [1929] and Aub *et al.* [1929] have made studies on the serum calcium and phosphorus in various disorders of the thyroid. There appears to be no unanimity among these various workers. Thus Aub and his colleagues, and McCullagh have reported that no changes occurred in the serum calcium and phosphorus in hypothyroidism or thyrotoxicosis. Most observers inclined to the view that the serum calcium was low in thyrotoxicosis although Aub and his collaborators [1929] have stated, 'the serum calcium and phosphorus values remained within the accepted normal limits, though a few cases suggested a slight fall'. Why the serum calcium should be low cannot readily be explained, occurring, as it does, during an excessive calcium mobilization and excretion. The serum calcium concentration is obviously the resultant of calcium added to the blood and calcium excretion. If these forces are equal, the serum calcium will remain constant, while if the excretion exceeds the calcium addition, then the concentration will fall. Following subtotal thyroidectomy Wade found that the serum calcium rose from an average of 8.3 to 10.8 mg. per 100 ml., but these findings differed from those of previous workers. It is known, of course, that if the parathyroids are removed along with the thyroid gland, tetany will develop and this will be accompanied by a sharp fall in the serum calcium. But a less marked fall in the serum calcium after thyroidectomy was detected in the absence of tetany, and this has led observers to postulate as a cause the occurrence of trauma to the parathyroid glands by manipulation during the operation or disturbance in the blood-supply from oedema.

Calcium balance experiments

The work of Voit [1880] established the fact that there could be a marked loss of calcium from the gut through endogenous metabolism. He fed animals on a very low calcium diet and found that the calcium excreted in the faeces was greater than the calcium intake. Rey [1895] extended these observations. He washed clean the gut of animals and then gave calcium subcutaneously and found that about 50% of the injected calcium was excreted into the intestine. Among the first observers to note the disparity between the calcium intake and output in exophthalmic goitre was Towles [1910]. She examined three cases of thyrotoxicosis on low and high calcium intakes and found a markedly negative balance in the former but a positive balance when large doses of calcium were given. On a low calcium diet the excretion of calcium in the urine exceeded the intake,

while the faecal loss was more than twice as much. Kummer [1917] confirmed that there was a high faecal excretion of calcium in exophthalmic goitre and attributed this to difficulty in absorption. Aub *et al.* [1929] made a most comprehensive study of the calcium and phosphorus metabolism on normal individuals and on those with thyroid disease or taking thyroid by mouth. On a low calcium intake of about 100 mg. a day they found normally in a three-day period that there was an excretion of 12.7 mg. of calcium per kg. of body-weight. In exophthalmic goitre on a similar diet they found a calcium excretion of 42.0 mg. per kg. and a similar, though not so well-marked, negative calcium balance was found in toxic adenoma. Further work by Michaud [1930], Hansman & Wilson [1934], Hansman & Carr-Fraser [1938], Puppel & Curtis [1936], Puppel, Klassen & Curtis [1939] and by Cope & Donaldson [1937] brought forward still more evidence that increased activity of the thyroid gland is associated with an increase in the excretion of calcium and phosphorus.

Post-mortem appearances of the skeleton

Von Recklinghausen performed the necropsy on a case dying of thyrotoxicosis under the care of Koeppen. Koëppen & von Recklinghausen [1891] and Koeppen [1892] published accounts of the case, and drew attention to the occurrence of the syndrome of thyrotoxicosis and decalcification of the skeleton leading to a condition of osteomalacia. The combination of osteomalacia and thyrotoxicosis was later drawn attention to by Latzko [1901] and Tolst & Sarvonat [1906]. The latter reported a single case, whereas Latzko in a series of 150 cases of osteomalacia found that 6 were thyrotoxic. Turnbull [1930], quoted by Hunter [1930], reported on the histological appearances of the bones in two cases of exophthalmic goitre and drew attention to the marked osteoporosis.

CAUSE OF THE DECALCIFICATION

The evidence therefore clinically, biochemically, radiographically and finally that based on post-mortem appearances of the skeleton leaves little doubt that in exophthalmic goitre a continuous decalcification is proceeding. Several theories of the causation of the marked calcium loss in thyrotoxicosis have been suggested and a certain controversy exists regarding the actual cause. The following causes have been advanced:

Increased basal metabolism per se

Aub *et al.* [1929], investigating this possibility, studied the calcium balance in cases with a high metabolism due to causes other than thyrotoxicosis, such as fever or leukaemia. Three such cases showed a normal calcium excretion, while the fourth case had a negative balance for calcium approaching that found in thyrotoxicosis of like elevated

metabolism. From their studies these authors concluded that an increased calcium excretion is not necessarily indicative of a raised basal metabolism.

Increased phosphorus excretion

Hocmicke [1904] suggested that the excessive loss of calcium in thyrotoxicosis was secondary to an increased excretion of phosphorus, the role played by the calcium being that of neutralizing phosphorus and other acid products. The cause of the excessive phosphorus excretion was attributed to the increased protein metabolism, one of the most prominent features of thyrotoxicosis.

Vitamin D deficiency

It is well known that vitamin D deficiencies are associated with a high output of calcium both in the urine and faeces, and the administration of vitamin D in adequate amounts has resulted in changing a negative to a positive calcium balance. Thus Gargill, Gilligan & Blumgart [1930] treated a case of osteomalacia with large doses of vitamin D, and the excessive calcium loss returned to normal. The similarity in the calcium loss between osteomalacia and thyrotoxicosis has led several investigators to postulate vitamin D deficiency as a causative factor in the latter disease. Rabinowitch [1929] compared the effect of iodine treatment with and without vitamins A and D on the basal metabolism in exophthalmic goitre and found that the average decrease with iodine was 3.2% daily, whereas with iodine and vitamins the decrease was 4.7%. He believed that the administration of large quantities of vitamins A and D influenced the course of exophthalmic goitre. Fraser & Cameron [1929] thought vitamins A and D alone were without effect on the course of thyrotoxicosis, and these findings were confirmed by Tibbits, McLean & Aub [1932], who found that large doses of irradiated ergosterol did not influence the greatly increased calcium excretion.

Direct stimulating catabolic effect of thyroxin on bone

Among others, Aub *et al.* [1929], Thomson & Collip [1932], Langdon-Brown [1932] and Cope & Donaldson [1937] are of the opinion that the increased excretion of calcium in thyrotoxicosis depends on a direct stimulating catabolic effect of the thyroid secretion on the calcium deposits in the bones. They have based their views on several observations that may be briefly summarized:

- (a) The serum calcium is normal.
- (b) The work of Parhon [1914], who fed rabbits on daily doses of thyroid varying from 50 to 300 mg. She noticed an excessive loss of calcium, the loss being greater as the dosage of thyroid increased.
- (c) The calcium loss is predominantly in the faeces.

- (d) The calcium excretion in myxoedema was found to be diminished, and after adequate treatment with thyroid or thyroxine had caused the basal metabolism to rise to normal the calcium excretion became normal.
- (e) In hypoparathyroidism the administration of thyroid or thyroxine resulted in an increase of the serum calcium. In a case of post-operative hypoparathyroidism recurrence of the thyrotoxicosis caused all signs of tetany to disappear, but with the administration of iodine and fall in the basal metabolism, tetany reappeared.

Coexisting hyperparathyroidism

Hansman & Wilson [1934] and Hansman & Carr-Fraser [1938] hold the view that the increased calcium excretion in thyrotoxicosis can only be explained by the presence of hyperparathyroidism, and support for their views may also be briefly summarized:

- (a) Iodine has caused the basal metabolism to fall to normal while the calcium excretion remained markedly excessive.
- (b) X-irradiation to the thyroid region changed the calcium balance from negative to positive, but clinically the thyrotoxicosis was as severe before X-ray therapy, and the basal metabolism remained as markedly increased above normal.
- (c) The use of diets higher in calcium than 0.2 g. daily has resulted in many positive balances in thyrotoxicosis.
- (d) Cases of hypoparathyroidism with coexisting toxic goitre have been described where there was calcium equilibrium.

Of the various causes which have been mentioned to account for the increased mobilization and excretion of calcium in thyrotoxicosis chief interest has centred around the last two.

It was with a view to determining more clearly, if possible, whether the calcium excretion depended on the direct action of thyroid secretion on bone or on an associated hyperparathyroidism that the following cases of thyrotoxicosis were studied.

EXPERIMENTAL METHODS

The ward routine and laboratory methods

The plans of the ward routine were based on those previously described by Bauer & Aub [1927]. The periods were of three days and before the observations were begun the subjects were put on the special diet, afterwards described, for two to three days. At the end of this preliminary stage, carmine alum lake 0.6 g. was given at 1 p.m., and again every three days at the same time. Usually the dye appeared in the faeces the follow-

ing morning, and this marked the beginning of the first period—i.e. the first stool containing the dye was not included in the first period.

Diet. The diet had a calcium content of 100–150 mg. daily, the acid and base being balanced. The daily calorific requirements were varied to suit individual requirements by the addition of foodstuffs very low in calcium content, the protein intake being in the region of 1 g. per kg. body-weight. The fluid and salt intake were kept constant. All utensils employed in the preparation of the food were washed with distilled water. The food was cooked by steaming, and eaten from the dish in which it was cooked. Variations in the calcium content of the articles of foodstuffs made the analysis of a duplicate diet essential. The extent of this variation is shown by the following figures for marmalade.

Calcium content of marmalade in g. per 100 g.:

Published value: 0.012

Value found: 0.071

Table I gives the published calcium content of the various foodstuffs used

Table I. *Calcium content of the diet*

Food or medicine	Calcium content (g. per. 100 g.)
<i>(a) Figures taken from Sherman [1937].</i>	
Apples	0.007
Bacon	0.006
Bananas	0.008
Beef (lean)	0.013
Bread (white)	0.031
Chicken	0.011
Honey	0.004
Lemon juice	0.022
Potatoes	0.013
Rice (uncooked)	0.011
Sugar	0.000
Tomatoes	0.011
<i>(b) Analyses carried out in Courtauld Institute</i>	
Ordinary salt	390 mg. per 100 g.
Digitalis tablet (gr. 1)	0.46 mg. per tablet
Barley sugar	8.0 mg. per 100 g.
Cascara Evacuant (Parke, Davis & Co.)	42.0 mg. per 100 ml.
Aerated soda water	0.06 mg. per 100 ml.
Haust. pot. brom.	2.3 mg. per fluid oz.
Pot. brom. gr. 15	
Syrup. aurant. m. 30	
Aq. chlorof. ad 1 oz.	
Thyroid tablet (gr. 2)	0.05 mg. per tablet.
Mist. iod. (60 mg. iodine in 1 oz.)	1.5 mg. per fluid oz.
Luminal tablet (gr. 1)	2.0 mg. per tablet

for the preparation of a low calcium diet, and our figures for the calcium content of any medicines taken by the patients as determined by us. But in all cases duplicate diets were prepared, every effort being made to

secure comparable samples—e.g. when meat from a joint was given to a patient an equal amount of meat from the same cut was used for the determination of calcium and phosphorus. Few, if any, of the patients or normal subjects complained about the nature of the diet and there was no loss of weight during its administration. Constipation occasionally occurred, and when a delay of 24 hours took place a simple distilled water enema was given.

Urine

The urine was collected in Winchester bottles in 24-hour specimens from 9 o'clock one morning to 9 o'clock the next. Toluene was used as a preservative, and the bottles were stored in a refrigerator by the bedside pending their removal to the laboratory.

Faeces

The stools were received into special bowls supplied with lids to fit, and these bowls were dispatched to the laboratory immediately.

Analyses of the specimens

The main difficulty in the analysis of a mixed diet or of faeces is that of obtaining a representative sample. The object in these experiments was to obtain a stable finely ground powder which permitted easy and accurate sampling. The presence of fat in faeces or in diet prevents fine subdivision of the dried material. For this reason the fat and at the same time a large proportion of the water were removed by treatment with acetone prior to drying and grinding. This process as applied to faeces was as follows: Each specimen of faeces, which had been passed directly into a large enamelled bowl, was treated with two volumes of acetone which was thoroughly mixed with the specimen. At the end of an hour all the specimens of the three-day period were filtered through a large Buchner funnel, using suction. The filtrate was measured and mixed with the three days' supply of urine or analysed separately, as desired. The residue on the filter-paper was warmed to expel the excess of acetone and then dried. When dry it was weighed, and afterwards ground to a fine powder in a mechanical grinder. In the case of the diet it was possible to omit the acetone extraction in most cases by separating the butterfat from the diet. In this case the three days' supply of butterfat was weighed, melted and well mixed prior to sampling. The diet was minced, dried at 105° C., weighed and then ground to a fine powder in a mechanical grinder.

Samples containing 6-20 mg. of calcium were used for the analysis. In the case of the diet, 30 g. of the powdered diet and its equivalent of butterfat were placed in the same dish. For urine usually 100 ml. were evaporated to dryness in a porcelain dish. The sample of faeces, usually

1 g., was weighed in a dish containing the equivalent of acetone extract of the faeces which had previously been evaporated to dryness. If the total excretion of calcium only was required, then a definite aliquot, e.g. 1/70th of the total quantity of urine, faeces and faecal extract, was measured into one dish and evaporated to dryness. The dishes were then placed overnight in a muffle furnace maintained at 500° C. Next day the ash was dissolved in hydrochloric acid and the solution was filtered, if necessary, and transferred to a conical flask.

Chemical methods

All the chemical determinations were done in duplicate. The calcium estimations of the diet, faeces and urine were determined by a modification of McCradden's method [1910, 1911], as described by Hawk & Bergeim [1938]. The precipitated calcium oxalate was collected on a Jena porous glass filter, and washed with dilute ammonia. The oxalate was then dissolved in dilute sulphuric acid and titrated with N/10 potassium permanganate solution. The phosphate was determined by the method of Fiske & Subbarow [1925] as modified by King [1932].

Basal metabolism

The basal metabolisms were measured by the closed circuit apparatus as described by Robertson [1937] and the standards of Means, Aub & Dubois [1917] employed.

EXPERIMENTAL RESULTS

Normal controls

Nine subjects, three men and six women, with ages varying from nineteen to fifty-four, were studied. All the subjects were normal, healthy individuals who had volunteered for the investigation. In all, 33 three-day periods were investigated, and each period was preceded by at least three days on a low calcium intake of 100 to 150 mg. daily. The data are presented in Table II, the subjects of each sex being arranged in order of their weights. It will be seen that a variation in the calcium and phosphorus excretion occurs among different individuals and also in different periods of the same individual. In all the cases studied there was one constant finding—a negative balance for calcium and phosphorus in every period. In the male subjects the average calcium intake per three days was 0.32 g. and the average output 0.73 g., resulting in an average negative balance of 0.41 g., whereas the average phosphorus intake per three-day period was 1.12 g., the average output 1.93 g., resulting in an average negative balance of 0.81 g. In the women controls, the corresponding figures for calcium were 0.31 g. and 0.78 g., resulting in an average negative balance of 0.47 g., and were for phosphorus 1.11 g. and 1.72 g., resulting in an average negative balance of 0.55 g. The results are similar to those of

previous investigators [Bauer, Albright & Aub, 1929; von Wendt, 1905; Sherman, Wheeler & Yates, 1918 and Sherman & Winters, 1918] whose experimental conditions were the same as those detailed above. From Table II it can be seen that the calcium excretion for a three-day period is 12.4 mg. per kilogram of body-weight for a man and 14.5 mg. for a woman, the phosphorus excretions being 32.9 mg. and 30.7 mg., respectively. It would appear therefore that the calcium loss is if anything greater in woman than in man.

Patients suffering from thyrotoxicosis

This series consisted of thirty-three consecutive cases of thyrotoxicosis admitted to the Courtauld Wards for investigation and treatment, comprising five men and twenty-eight women. Two cases, Nos. 1 and 8 (a man and a woman), were too ill to allow a study of the calcium and phosphorus metabolism in the pre-iodine stage and the later investigations were not carried out as they would have been of little relative importance. It was endeavoured to make a study of the calcium and phosphorus balance in the active stage of the thyrotoxicosis; by that is meant at a time when either no iodine had ever been taken, or when iodine had not been taken for a period of at least two months. Afterwards in the text we shall refer to this stage as the 'iodine-fast' stage, indicating that thyrotoxicosis was not controlled by any action of iodine and was therefore in its most active stage. The balance was again studied during the maximum beneficial effect of iodine therapy as judged by frequent basal metabolism measurements. During this stage the B.M.R. was constant and level, for a period of at least ten days, and the calcium and phosphorus balance was studied in the middle of this period. By this means it was endeavoured to find out whether the calcium and phosphorus loss fell *pari passu* with that of the basal heat output, the latter being constant over a sufficiently long period to make one comparable with the other. The final observation was made two to three weeks after subtotal thyroidectomy at a time when the B.M.R. was again at a constant level for a period of at least seven to ten days. Unfortunately three specimens after iodine therapy were lost (Nos. 2, 22 and 28). In Table III are given the figures for the calcium and phosphorus balance in 31 of 33 cases of thyrotoxicosis studied (2 cases were too ill for study). The cases are arranged in order of their toxicity as determined by the level of the basal metabolism in the untreated or 'iodine-fast' stage. In Table IV are given the data on the calcium and phosphorus excretion per kilogram of body-weight before and during iodine therapy and after subtotal thyroidectomy.

To give a general impression four scatter diagrams are included (Figs. 1-4). Figs 1 and 2 are scatter diagrams relating the basal metabolic rate

Table II. *Calcium and phosphorus metabolism in normal subjects*

No.	Name	Age	Wt. (kg.)	Calcium in g. per 3-day period			Total calcium excretion	Phosphorus in g. per 3-day period			Total phosphorus excretion
				Intake	Out- put	Balance	per kg. (mg.)	Intake	Out- put	Balance	per kg. (mg.)
(a) <i>Males</i>											
1	S.W.	27	57.3	0.31	0.72	-0.41	12.6	1.05	2.07	-1.02	36.1
				0.20	0.95	-0.66	16.6	1.10	2.42	-1.32	42.2
				0.20	0.67	-0.38	11.7	1.06	2.09	-1.03	36.5
				0.37	0.72	-0.35	12.5	1.10	2.21	-1.11	38.5
2	A.K.	30	58.7	0.39	0.85	-0.46	14.5	1.01	1.77	-0.76	30.1
				0.37	0.74	-0.37	12.6	1.07	1.72	-0.65	29.3
				0.32	0.74	-0.42	12.6	1.07	1.63	-0.56	27.8
				0.30	0.69	-0.39	11.7	1.11	1.50	-0.39	25.5
				0.26	0.89	-0.63	15.1	1.10	1.81	-0.71	30.8
				0.27	0.84	-0.57	14.3	1.08	1.73	-0.65	29.4
3	A.D.	54	60	0.27	0.45	-0.18	7.5	1.15	1.97	-0.82	32.8
				0.38	0.55	-0.17	9.2	1.28	1.95	-0.67	32.5
				0.37	0.87	-0.50	14.5	1.24	2.41	-1.17	40.1
				0.33	0.51	-0.18	8.5	1.27	1.73	-0.46	28.8
Average			0.32	0.73	-0.41	12.4	1.12	1.93	-0.81	32.9	
Range			0.26	0.45	-0.17	7.5	1.01	1.50	-0.39	25.5	
			to	to	to	to	to	to	to	to	
				0.39	0.95	-0.66	16.6	1.28	2.42	-1.32	42.2
(b) <i>Females</i>											
1	M.C.	50	42.7	0.32	0.90	-0.58	21.0	1.09	1.67	-0.58	39.1
				0.25	0.72	-0.47	16.8	0.94	1.40	-0.46	32.8
				0.25	0.71	-0.46	16.6	1.04	1.41	-0.37	33.0
2	D.K.	29	43.6	0.39	1.01	-0.62	23.2	1.24	1.98	-0.74	45.4
				0.39	0.72	-0.33	16.5	1.20	1.90	-0.70	43.6
				0.30	0.97	-0.67	22.2	1.03	1.87	-0.84	42.9
				0.34	0.67	-0.33	15.4	1.22	1.49	-0.27	34.2
3	D.S.	19	51.8	0.38	0.58	-0.20	11.2	1.28	1.30	-0.02	25.1
4	R.S.	48	57.8	0.26	0.65	-0.39	11.2	1.10	1.83	-0.73	31.6
				0.27	0.84	-0.57	14.5	1.08	1.75	-0.67	30.3
				0.26	0.89	-0.63	15.4	1.10	1.74	-0.64	30.1
				0.26	0.94	-0.68	16.2	1.10	1.61	-0.51	27.8
5	H.B.	36	58.2	0.25	0.53	-0.28	9.1	1.12	1.55	-0.43	26.6
6	A.S.	32	71.7	0.45	0.79	-0.34	11.0	1.12	1.94	-0.82	27.0
				0.40	0.86	-0.46	12.0	1.05	1.91	-0.86	26.6
				0.34	0.72	-0.38	10.0	1.11	1.60	-0.49	22.3
				0.30	0.70	-0.40	9.8	1.10	1.28	-0.18	17.8
				0.26	0.86	-0.60	12.0	1.10	1.74	-0.64	24.2
				0.27	0.76	-0.49	10.6	1.08	1.64	-0.56	22.8
Average			0.31	0.78	-0.47	14.5	1.11	1.72	-0.55	30.7	
Range			0.25	0.53	-0.20	9.1	0.94	1.28	-0.02	17.8	
			to	to	to	to	to	to	to	to	
				0.45	1.01	-0.68	23.2	1.28	1.98	-0.86	45.4

to the concurrent level of calcium and phosphorus balance respectively. Figs. 3 and 4 show the relation between the percentage fall in basal metabolic rate following iodine therapy or operation and the percentage change in calcium and phosphorus balance at the same times; in both cases a rise

Table III. *Cases of thyrotoxicosis*

Name	Wt. in kg.			B.M.R.			Calcium balance in g.			Phosphorus balance in g.			
	Before	On	After	Before	On	After	Before	On	After	Before	On	After	
		iodine	oper.		iodine	oper.		iodine	oper.		iodine	oper.	
G.B.	56.5	52.3	52.3	+67	+30	+3	—	—	—	—	—	—	1
G.T.	66.2	—	71.7	+60	+14	-8	2.68	—	0.79	1.12	—	1.19	2
E.G.	50.9	50.3	50.4	+61	+7	-19	0.64	0.46	0.30	0.36	0.18	0.17*	3
E.D.	43.2	43.8	44.4	+57	-4	-21	1.61	0.35	0.14	0.72	0.26	0.34	4
C.N.	51.3	50.9	50.5	+46	+9	+1	0.93	0.05	0.03	0.84	0.45	0.31	5
G.H.	59.3	59.3	58.6	+46	0	-19	1.63	1.75	0.58	0.55	0.82	0.23	6
E.S.	49.3	54.1	55.2	+43	+5	-3	1.69	1.20	0.19	0.79	0.81	0.45	7
A.C.	38.7	37.5	37.5	+43	+9	-1	—	—	—	—	—	—	8
V.C.-H.	49.5	51.8	53.4	+42	+2	-6	1.28	0.95	0.48	1.13	0.06	0.52	9
M.E.	41.8	41.5	44.4	+42	+5	+3	1.21	0.67	0.60	0.83	0.49	0.50	10
L.R.	44.8	46.4	47.7	+38	+19	-4	1.06	0.64	0.46	0.61	0.91	0.16	11
G.J.	57.0	58.0	58.1	+37	-6	-8	1.10	1.12	0.48	1.08	1.35	0.19	12
E.J.	56.4	57.5	57.5	+36	-4	-14	0.62	0.30	0.84	0.40	0.01	0.59	13
E.H.	46.6	47.8	48.0	+29	+7	-17	1.07	0.49	0.30	0.95	0.29	0.62	14
E.H.	45.9	50.3	50.0	+29	-3	-15	1.06	0.69	0.49	1.16	0.75	0.51	15
A.S.	50.9	51.5	53.3	+28	+8	-6	0.72	0.59	0.41	0.55	1.03	0.28	16
E.R.	61.5	59.8	59.8	+27	+1	-17	0.99	0.80	1.22	0.96	0.57	0.61	17
G.F.	48.0	51.1	53.6	+27	+8	-14	1.91	1.11	0.38	1.67	1.30	0.22	18
J.S.	45.5	46.8	50.0	+24	-3	-14	0.66	1.08	1.43	0.92	1.17	0.88	19
L.P.	43.2	43.6	43.6	+21	+2	+3	0.39	0.37	0.32	0.08	0.26	0.01	20
N.H.	57.5	61.1	61.1	+20	-4	-15	0.52	0.69	0.59	0.95	0.55	0.20	21
C.W.	46.0	47.0	47.0	+20	-11	-4	0.41	—	0.32	0.34	—	0.36	22
W.T.	52.5	53.0	51.9	+20	+4	-10	0.57	0.19	0.29	0.16	0.01	0.07	23
E.F.	53.7	54.5	55.0	+20	+9	-4	1.81	1.70	0.46	1.27	0.85	0.02	24
F.M.	79.0	79.1	76.5	+19	-2	-1	0.78	1.13	0.17	1.05	0.48	0.01	25
M.R.	50.9	52.0	49.0	+18	+13	-4	0.53	0.77	0.48	0.33	1.09	0.08	26
J.W.	48.3	49.0	51.3	+16	+4	0	1.32	1.26	0.59	0.85	0.82	0.06	27
F.W.	54.5	57.2	57.2	+15	+8	-9	1.08	—	0.07	1.14	—	0.28	28
B.R.	50.0	50.4	52.2	+15	-14	-24	1.53	1.67	0.15	0.48	0.67	0.78	29
M.C.	57.7	56.8	52.8	+14	-4	-25	1.01	1.59	0.06	0.62	1.18	0.32	30
L.H.	41.3	42.0	40.7	+7	-2	-6	0.68	0.49	0.51	0.68	0.67	0.47	31
J.T.	56.2	59.7	59.9	+6	-22	-27	0.12	0.22	0.37	0.64	0.71	0.59	32
E.K.	42.1	42.2	42.0	+1	-9	-17	0.85	0.66	0.67	0.18	0.17	0.19	33

* All balances are negative with the exception of those in Clarendon type

in the negative balance is regarded as a positive change. If there is a direct relation the points in the graphs should fall along a straight line. The degree of scatter is, however, very great and appears to us to indicate that the relationship is not direct. This conclusion is supported by the consideration of the individual results given below.

Calcium metabolism

Calcium excretion within normal limits

In 15 of the 33 cases the calcium excretion in mg. per kg. was within or almost within the limits of the normal controls given in Table II, although

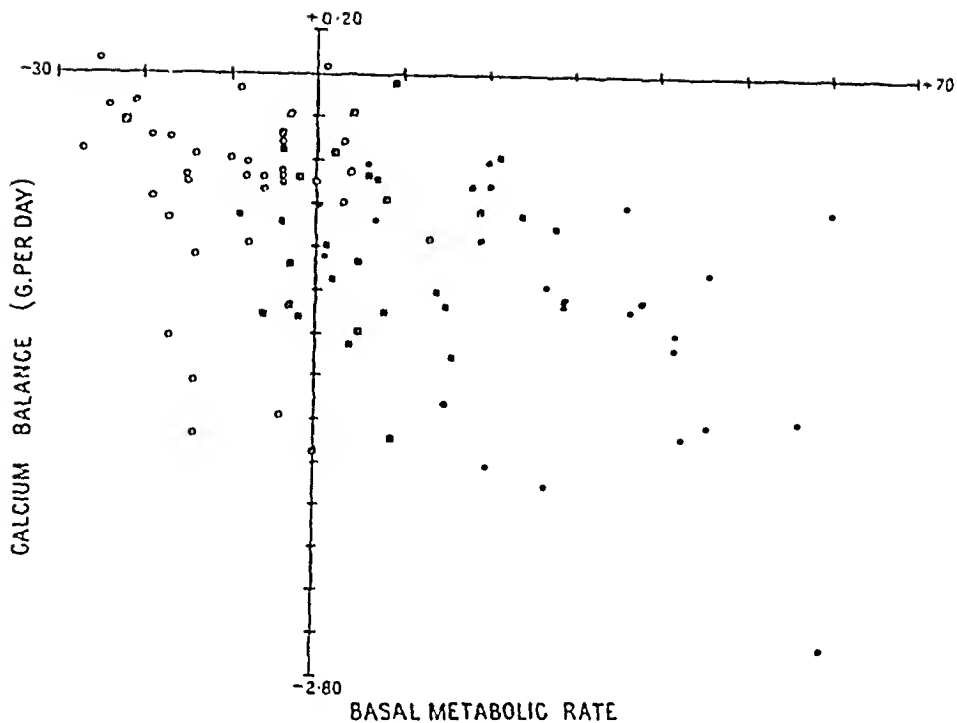


FIG. 1. Relation between calcium balance and basal metabolic rate.

- Cases of thyrotoxicosis
- ◻ " " treated with iodine
- " " after subtotal thyroidectomy.

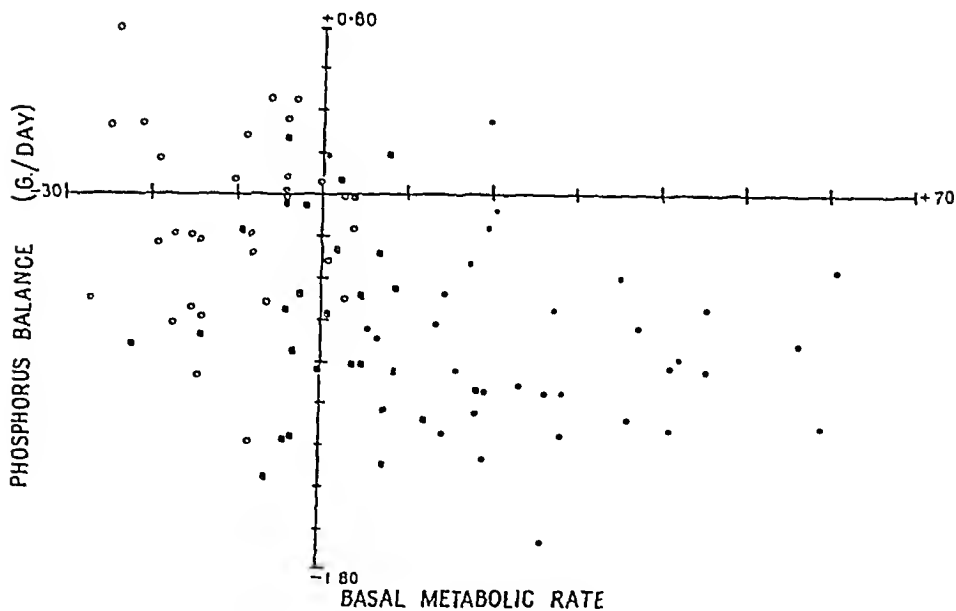


FIG. 2. Relation between phosphorus balance and basal metabolic rate.

- Cases of thyrotoxicosis
- ◻ " " treated with iodine.
- " " after subtotal thyroidectomy.

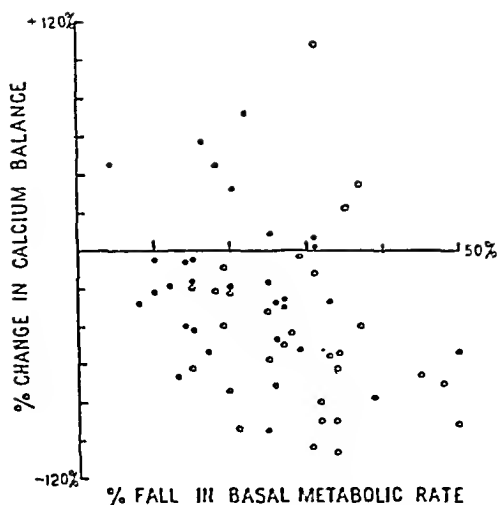


FIG. 3. Relation between fall in basal metabolic rate and change in calcium balance when thyrotoxic patients are treated with iodine (●) or by subtotal thyroidectomy (○).

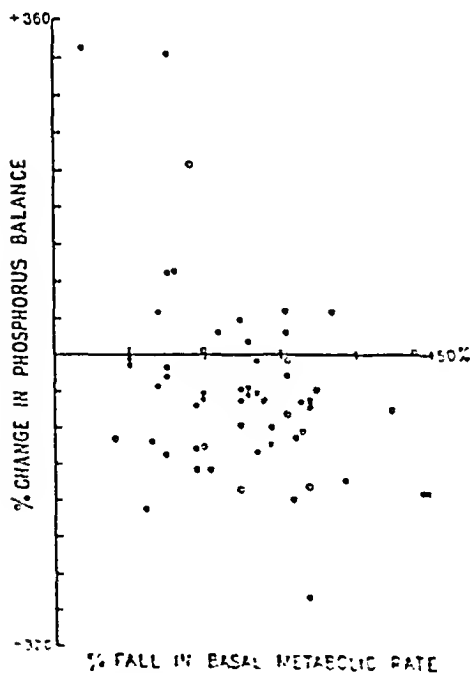


FIG. 4. Relation between fall in basal metabolic rate and change in phosphorus balance when thyrotoxic patients are treated with iodine (●) or by subtotal thyroidectomy (○).

the range of the 'iodine-fast' basal metabolisms varied from +1 to +67 with a mean figure of +30. Iodine medication brought about a fall in the basal metabolisms of all these cases, but a study of the calcium metabolism showed that a diminution in the calcium excretion occurred in 7 or 54%

Table IV. *Calcium and phosphorus excretion in thyrotoxicosis*

No.	Name	Calcium excretion in mg. per kg. of body-wt. per 3-day period			Phosphorus excretion in mg. per kg. of body-wt. per 3-day period			Type of gland
		Before	On iodine	After oper.	Before	On iodine	After oper.	
1	G.B.	—	—	—	—	—	—	Diffuse
2	G.T.	46.2	—	15.9	42.8	—	39.0	Nodular
3	E.G.	19.2	15.9	12.1	41.9	35.0	29.4	Nodular
4	E.D.	42.8	13.5	8.8	47.2	25.3	24.8	Diffuse
5	C.N.	24.9	9.0	7.3	46.5	40.9	37.6	Nodular
6	G.H.	32.2	33.7	14.3	35.4	39.4	30.4	Nodular
7	E.S.	39.6	27.7	8.2	52.9	50.3	27.0	Nodular
8	A.C.	—	—	—	—	—	—	Diffuse
9	V.C.-H.	32.3	23.3	13.3	64.7	42.8	39.5	Nodular
10	M.E.	36.1	27.0	19.6	48.8	43.6	40.9	Nodular
11	L.R.	29.0	19.4	14.3	50.9	54.9	35.2	Diffuse
12	G.J.	24.6	25.2	12.7	47.4	37.6	28.8	Diffuse
13	E.J.	16.3	10.2	19.1	36.8	29.2	36.7	Diffuse
14	E.H.	29.1	15.4	11.5	50.0	35.4	41.8	Nodular
15	E.H.	28.7	22.9	15.4	54.2	43.9	38.2	Diffuse
16	A.S.	20.1	17.9	13.5	45.9	46.2	37.1	Diffuse
17	E.R.	20.5	17.9	26.1	47.0	36.0	38.8	Diffuse
18	G.F.	44.2	24.8	12.1	70.7	55.3	34.1	Nodular
19	J.S.	19.8	29.2	33.8	49.4	57.9	48.7	Diffuse
20	L.P.	14.0	13.8	13.8	37.4	43.0	38.5	Nodular
21	N.H.	13.4	15.4	13.1	41.3	31.2	26.5	Nodular
22	C.W.	16.7	Lost	13.6	32.0	Lost	30.6	Diffuse
23	W.T.	15.4	8.5	13.1	28.4	24.0	24.6	Diffuse
24	E.F.	40.6	37.6	13.3	55.5	46.7	28.0	Nodular
25	F.M.	13.9	18.2	9.9	33.4	27.7	19.9	Nodular
26	M.R.	14.9	18.7	14.3	34.0	47.8	28.0	Nodular
27	J.W.	34.7	33.9	16.4	51.8	52.6	30.4	Diffuse
28	F.W.	24.1	—	5.3	49.0	—	21.6	Nodular
29	B.R.	34.0	37.9	7.9	36.4	42.0	13.0	Diffuse
30	M.C.	21.6	33.3	4.0	40.5	50.7	22.9	Diffuse
31	L.H.	23.5	18.6	18.7	50.7	36.6	25.6	Nodular
32	J.T.	11.9	8.2	10.7	38.6	37.5	34.5	Diffuse
33	E.K.	28.2	21.8	23.6	34.0	40.2	39.3	Diffuse

of the 15 cases (in 2 cases—Nos. 32, 38—the specimens after iodine were lost). In other words, although iodine brought about a remission in the symptoms of the thyrotoxicosis and a fall in the level of the basal metabolism in all the patients, in 6 or 46% the calcium excretion remained unchanged from or rose above the 'iodine-fast' rate.

Calcium excretion increased above normal

In 18 of the 33 cases the calcium excretion was greater than normal. The range of the basal metabolisms of these patients varied from +1 to

+60 with a mean figure of +35. There was therefore little difference in the severity of the thyrotoxicosis as judged by the basal metabolism in these two groups of cases, yet in one the calcium mobilization and excretion was above normal and in the other within normal limits. The calcium excretion was studied after the maximum beneficial response to iodine in 15 cases (Nos. 2 and 28 specimens lost), and in 11 or 73% the calcium excretion diminished as the basal metabolism fell, whereas in the remaining 27% the calcium excretion remained unchanged or was increased still more.

A study of the calcium excretion in the untreated 'iodine-fast' stage of the thyrotoxicosis gives further evidence that there is no relationship between the mobilization and excretion of calcium and the degree of increase of the B.M.R. Thus in case 3 for example, the B.M.R. was +61 yet the calcium excretion was well within normal limits and clinically, apart from the B.M.R. studies, this was a case of very severe thyrotoxicosis. Three other cases not included in the table, as post-operative tetany interfered with the calcium and phosphorus studies, had B.M.R.'s of +79, +54 and +49, indicating a severe degree of thyrotoxicosis, yet their calcium excretion was within normal limits being 19.5, 14.0 and 22.4 mg. per kg. of body-weight per three-day period.

With the exception of two cases (Nos. 17 and 19) the calcium excretion after subtotal thyroidectomy was within or almost within normal limits. In seven cases, however, the calcium excretion after operation was either unchanged from or was greater than the pre-iodine rate. Table V gives the particulars of these cases. Both clinically and from a study of their basal metabolism these patients were cured of their thyrotoxicosis by subtotal thyroidectomy; nevertheless the excretion of calcium remained unchanged from, or was greater than the rate of calcium loss when the thyrotoxicosis was active.

Table V

Number	Basal metabolism		Calcium excretion (mg./kg.)	
	Before oper.	After oper.	Before oper. and iodine	After oper.
13	+36	-14	16.3	19.1
17	+27	-17	20.5	26.1
19	+24	-14	19.8	33.8
20	+21	+3	14.0	13.8
21	+20	-15	13.4	13.1
26	+18	-4	14.9	14.3

Phosphorus metabolism

Phosphorus excretion within normal limits

In 15 of the 33 cases the phosphate excretion was within the normal limits of 17.8 to 45.4 mg. per kg. of body-weight per three-day period.

Usually a normal phosphorus excretion was associated with a normal calcium excretion, but in some cases where the phosphorus excretion lay within normal limits the calcium loss was greater than normal and vice versa. Iodine medication brought about a fall in 5 of the 13 cases studied (39%) (Nos. 2 and 22 specimens lost), and in the remaining eight (61%) the phosphorus excretion either remained unchanged or became greater, although symptomatic improvement and a fall in the basal metabolism occurred in all cases.

Phosphorus excretion increased above normal

In 16 of the 33 cases the phosphate excretion was greater than normal. Iodine medication brought about a diminution in 12 cases, in 4 cases the excretion remained practically unchanged or increased.

As in the case of the calcium metabolism no correlation was found between the severity of the thyrotoxicosis and the degree of phosphate loss.

SUMMARY

1. The calcium and phosphorus balances have been studied in normal men and women on a low intake of these minerals (calcium 100–150 mg. daily; phosphorus 350–450 mg. daily). A negative balance for calcium and phosphorus was constantly found:

Three-day period observations

	Calcium metabolism				Phosphorus metabolism			
	Negative balance in g.		Excretion in mg. per kg.		Negative balance in g.		Excretion in mg. per kg.	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Men	0.41	0.17–0.66	12.4	7.5–16.6	0.81	0.39–1.32	32.9	25.5–42.2
Women	0.47	0.20–0.68	14.5	9.1–23.2	0.55	0.02–0.86	30.7	17.8–45.4

2. The calcium and phosphorus metabolism has been studied in a series of 33 cases of thyrotoxicosis on a low intake of these minerals similar to that ingested by the normal controls. A negative balance for calcium and phosphorus was constantly found, but the average loss was greater than in the series of normal people.

On iodine medication a symptomatic improvement occurred in all cases, the basal metabolism fell, and the mean calcium and phosphorus excretion also diminished.

After subtotal thyroidectomy a further fall in the basal metabolism invariably occurred and this was accompanied by a still further diminution in the mean calcium and phosphorus excretion. These were the findings in the series of cases which were clinically and biochemically free from post-operative tetany.

3. The above evidence would appear to favour the theory that the increased calcium and phosphorus loss in thyrotoxicosis is due to the direct effect of thyroxine on bones, assuming that there is an excessive

	Calcium excretion in mg. per kg. in 3 day period		Phosphorus excretion in mg. per kg. in 3 day period		Basal metabolism	
	Mean	Range	Mean	Range	Mean	Range
Before treatment	26.2	46.2-11.9	45.0	70.0-28.4	+30	+67 to +1
After iodine therapy	21.4	37.9-8.2	41.2	57.9-24.0	+2	+30 to -22
After operation	14.0	33.8-4.0	32.0	48.7-13.0	-10	+4 to -27

secretion of thyroxine in that disease. Closer study, however, indicates that this theory is not a satisfactory explanation. Thus:

- In almost half of the cases studied the calcium and phosphorus excretion was within the limits of the normal controls. This in itself is not a strong argument as it is recognized that if the normal range is wide, overlapping of normal and abnormal findings may occur.
- There was no correlation between the severity of the thyrotoxicosis as judged clinically (and confirmed by the basal metabolism) and the calcium and phosphorus loss, e.g. the severest case of thyrotoxicosis had a normal mineral metabolism.
- Although iodine caused a remission in all cases with a fall in the level of the basal metabolism, in only 55% of cases did the calcium and phosphorus excretion diminish.
- In six cases the calcium excretion remained the same after as before successful operation. One of these cases had a pre-operative basal metabolism of +36 and a post-operative one of -14.

These findings suggest that some other cause than the direct action of thyroxine secretion on bone must be sought to account for the decalcification and the excessive calcium and phosphorus loss from the body in thyrotoxicosis.

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THE TIME AND RATE OF APPEARANCE OF GONADOTROPHIN IN THE SERUM OF PREGNANT MARES

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APART from the work of Cole and his collaborators, little is known about the rate of production and the maximal concentration of the gonadotrophin in the serum of pregnant mares. Cole & Hart [1930] showed that the hormone first appears in the blood between the 5th and 6th week and that maximal concentration is attained between the 60th and the 90th day. In a later paper Cole [1938] observed that the peak concentration of gonadotrophin in the serum of pregnant ponies was four times as great as it was in the blood of mares of larger breeds, and also that it occurred for an even shorter time. The importance of accurate information about the time and duration of peak concentration has led us to obtain data for breeds of ponies available in England.

We have also investigated the extent to which the pregnant mare can be bled on one occasion, and the frequency with which large volumes of blood can be withdrawn, without affecting the concentration of the hormone and without interfering with the course of pregnancy.

MATERIAL AND METHODS

Production and determination of pregnancy

Eight mares, lettered A-H, were used. Six of these (A-F) were Welsh ponies, one (G) was a New Forest pony and one (H) a Shetland pony. They were inseminated at various times in oestrus with sperm collected from a stallion of unknown breed. The ejaculate was collected in an artificial vagina. The seminal fluid was separated into accessory secretions and sperm, and the mares inseminated with the latter. The occurrence of ovulation was determined by palpation of the ovaries, *per rectum*. On the average, ovulation occurred about 48 hours after insemination. Pregnancy is dated from the day of ovulation. All mares were diagnosed as pregnant by rectal palpation prior to the commencement of bleeding [Day, 1940].

Collection of blood and serum

Blood was collected in open beakers by the introduction of a fairly wide-bore needle into the jugular vein, and was allowed to stand at room temperature for 24 hours. The serum was then decanted and centrifuged, and 24 hours later, on its arrival at Hampstead, it was cold-stored.

Storage of serum

After a small sample of the serum had been taken for assay purposes, the remainder of each of the large volumes of serum was distributed in ampoules of 15 ml. and in bottles containing 100 ml. The serum was then dried in high vacuo by the method of Greaves & Adair [1938]. Assays of the dried serum have shown that no loss in activity occurs during this process of drying.

Biological assay

The serum was assayed by its ability to increase the weight of the ovaries of immature rats. Groups of 5 or 10 rats weighing 40–50 g. were injected subcutaneously once daily for 5 days. The animals were killed 24 hours after the last injection, the ovaries and uterus were dissected and fixed in Bouin's fluid overnight. On the following day these organs were weighed from 70% alcohol. The activity of each sample of serum was calculated in International Units (I.U.) of mare serum gonadotrophin by translating the response obtained into I.U. by means of a dose-response curve for the International Standard preparation [Rowlands & Williams, 1941]. Assays on the International Standard preparation which were carried out during the course of these investigations agreed closely with the dose-response curve.

RESULTS

The data concerning the times of bleeding and the activity of the serum obtained at different stages during early pregnancy are given for each mare in separate tables (Tables I–VIII), and in Figs. 1–3.

Mare A. Welsh pony; age 6 years. Commencing on the 47th day of pregnancy, one litre of blood was withdrawn every 14 days until the 132nd day, except that nearly two litres were withdrawn on the 104th day. Data and results are given in Table I.

Mare B. Welsh pony; age 5 years; weight 460 lb. Commencing on the 30th day of pregnancy, amounts of blood varying between 200 and 280 ml. were withdrawn every 14 days until the 142nd day, when the mare slipped her foal. It is extremely unlikely that abortion was caused by the withdrawal of these quantities of blood. Data are given in Table II.

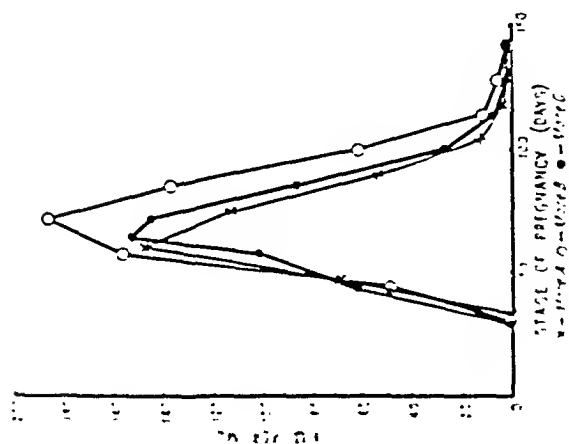


FIG. 1.

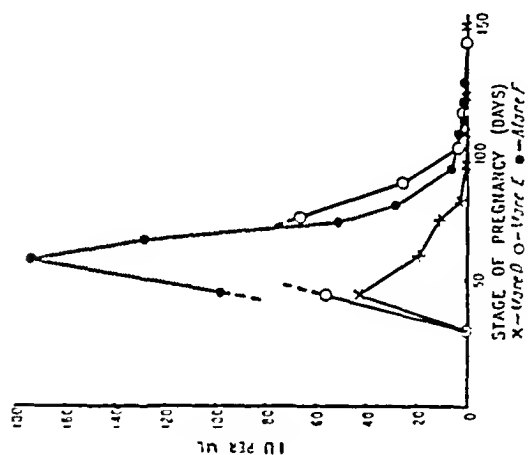


FIG. 2.

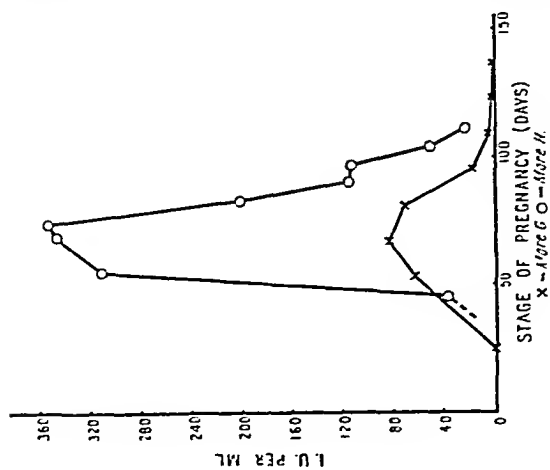


FIG. 3.

FIGS. 1-3. The content of gonadotrophin in International Units (I.U.) per ml. in the serum of eight mares (A-H) during early pregnancy.

Mare C. Welsh pony; age 6 years; weight about the same as *Mare B.* Bled every 14th day from the 30th day of pregnancy. At the time of maximal activity 1000 ml. of blood were withdrawn and 7 and 21 days later 3000 ml. were obtained. Data are given in Table III.

Table I. *Mare A*

Collection of serum			Biological assay			
No. of sample	Stage of pregnancy (days)	Amount of blood (ml.)	No. of rats	Amount of serum injected (ml.)	Weight of ovaries (mg.)	I.U. per litre of serum
A ₁	33	250	5	10	11	0
A ₂	47	1000	5	0.25	29	69,000
A ₃	61	1000	5	0.25	67	147,000
A ₄	75	1000	5	0.125	25	112,000
A ₅	90	1000	10	0.25	24	53,000
A ₆	104	1800	5	0.5	16	12,000
A ₇	118	1000	5	2.5	16	2,400
A ₈	132	1000	5	5.0	13	800

Table II. *Mare B*

Collection of blood			Biological assay			
No. of sample	Stage of pregnancy (days)	Amount of blood (ml.)	No. of rats	Amount of serum injected (ml.)	Weight of ovaries (mg.)	I.U. per litre of serum
B ₁	30	250	5	10	12	0
B ₂	44	200	5	0.25	23	48,000
B ₃	58	200	10	0.25	83	156,000
B ₄	72	220	5	0.25	99	186,000
B ₅	86	280	10	0.25	71	136,000
B ₆	100	250	10	0.25	26	61,000
B ₇	114	270	10	1.0	21	11,000
B ₈	128	200	5	2.5	23	5,000
B ₉	142	200	5	5.0	17	1,500

Table III. *Mare C*

Collection of blood			Biological assay			
No. of sample	Stage of pregnancy (days)	Amount of blood (ml.)	No. of rats	Amount of serum injected (ml.)	Weight of ovaries (mg.)	I.U. per litre of serum
C ₁	30	200	5	10	11	0
C ₂	44	250	5	0.25	27	62,000
C ₃	58	200	5	0.25	41	101,000
C ₄	65	1000	5	0.25	71	152,000
C ₅	72	3000	5	0.125	30	144,000
C ₆	86	3000	5	0.125	22	86,000
C ₇	100	200	10	0.5	24	26,000
C ₈	114	250	10	2.5	28	6,600
C ₉	128	250	5	5.0	21	2,000
C ₁₀	142	200	5	5.0	15	1,000

Mare D. Welsh pony; age 5 years; weight about the same as Mare B. Small amounts of blood were withdrawn at fortnightly intervals from the 30th to the 151st day of pregnancy except for two large samples of 1300 ml. and 2500 ml. which were taken on the 74th and 81st day respectively. The serum contained relatively small quantities of gonadotrophin; maximal concentration occurred on the 44th day. It is unlikely that the amount of blood withdrawn was responsible for the low 'peak' of activity. Data are given in Table IV.

Table IV. *Mare D*

Collection of blood			Biological assay			
No. of sample	Stage of pregnancy (days)	Amount of blood (ml.)	No. of rats	Amount of serum injected (ml.)	Weight of ovaries (mg.)	I.U. per litre of serum
D ₁	30	200	5	10	11	0
D ₂	44	200	5	0.25	21	43,000
D ₃	60	200	5	0.5	19	18,500
D ₄	74	1300	5	1.0	22	11,000
D ₅	81	2500	5	2.5	16	2,400
D ₆	96	250	5	5.0	12	0
D ₇	109	200	5	5.0	11	0
D ₈	123	200	5	5.0	10	0
D ₉	137	200		Not tested		
D ₁₀	151	200	5	5.0	9	0

Mare E. Welsh pony; age 3 years; much smaller than Mares B, C or D. Commencing on the 30th day of pregnancy, small amounts (200-300 ml.) of blood were withdrawn every 14 days until the 145th day, with the exception of one very large bleeding (3000 ml.) on the 75th day. Data are given in Table V. The loss of one sample (E.3) on the 61st day of pregnancy made it impossible to assay the serum at the time when the maximal concentration of the hormone was expected.

Table V. *Mare E*

Collection of blood			Biological assay			
No. of sample	Stage of pregnancy (days)	Amount of blood (ml.)	No. of rats	Amount of serum injected (ml.)	Weight of ovaries (mg.)	I.U. per litre of serum
E ₁	30	200	5	10	12	0
E ₂	44	200	5	0.25	25	56,000
E ₃	61	200		Bottle broken in transit		
E ₄	75	3000	5	0.25	28	66,000
E ₅	89	300	5	0.5	24	25,000
E ₆	103	200	5	5.0	30	3,500
E ₇	117	250	5	5.0	18	1,500
E ₈	131	250		Sample not tested		
E ₉	145	200	5	5.0	9	0

Mare F. Welsh pony; age 9 years. During the period of maximal

activity 2000 ml. of blood withdrawn weekly. This was followed by fortnightly bleedings of 200 ml. Data are given in Table VI.

Table VI. *Mare F*

Collection of blood			Biological assay			
No. of sample	Stage of pregnancy (days)	Amount of blood (ml.)	No. of rats	Amount of serum injected (ml.)	Weight of ovaries (mg.)	I.U. per litre of serum
F ₁	31	250	—	—	—	—
F ₂	45	200	5	5	108	98,000
F ₃	59	2000	10	0.125	35	173,000
F ₄	66	2000	10	0.1	24	128,000
F ₅	73	2000	10	0.25	24	51,000
F ₆	80	2000	10	1.0	45	28,000
F ₇	94	200	5	2.5	27	6,000
F ₈	108	200	5	5.0	26	3,000
F ₉	114	200	5	5.0	14	900
F ₁₀	122	200	5	5.0	18	1,600
F ₁₁	129	200	5	5.0	15	1,000

Mare G. New Forest pony; age 5 years; weight 640 lb. Commencing on the 25th day of pregnancy small amounts of blood (200–250 ml.) were withdrawn at fortnightly intervals until the 67th day (inclusive). This was followed by two large bleedings (3000 ml.) at 81 and 95 days. Data are given in Table VII.

Table VII. *Mare G*

Collection of blood			Biological assay			
No. of sample	Stage of pregnancy (days)	Amount of blood (ml.)	No. of rats	Amount of serum injected (ml.)	Weight of ovaries (mg.)	I.U. per litre of serum
G ₁	25	200	5	10.0	10	0
G ₂	39	250	5	0.25	10	?
G ₃	53	200	10	0.5	53	63,000
G ₄	67	200	5	0.25	34	84,000
G ₅	81	3000	5	0.125	20	70,000
G ₆	95	3000	10	0.5	19	17,000
G ₇	109	250	10	2.5	21	4,000
G ₈	123	250	5	5.0	19	2,000
G ₉	137	200	5	5.0	13	800

Mare H. Shetland pony; age about 18 years; weight 500 lb.—this animal was very fat. The scheme of bleeding was similar to that for Mare F, with the exception that blood was withdrawn at weekly intervals throughout until the 111th day of pregnancy. Data are given in Table VIII.

SUMMARY

1. Gonadotrophin appeared in the serum of pregnant ponies between the 30th and 47th day after ovulation and very quickly reached its

maximal concentration, which is maintained for a short period between the 60th and 75th day.

2. During this period the serum of a Shetland pony (Mare H) contained 352,000 I.U. per litre; that of four of the six Welsh ponies (Mares A, B, C and F) between 150,000 and 185,000 I.U. per litre; that of a New Forest pony (Mare G) and that of another Welsh pony (Mare D) contained a maximal of 84,000 and 43,000 I.U. per litre respectively.

3. Gonadotrophin had almost completely disappeared by the 110th day.

Table VIII. *Mare H*

Collection of blood			Biological assay			
No. of sample	Stage of pregnancy (days)	Amount of blood (ml.)	No. of rats	Amount of serum injected (ml.)	Weight of ovaries (mg.)	I.U. per litre of serum
H ₁	45	200	10	0.25	20	37,000
H ₂	55	2000	10	0.125	74	310,000
H ₃	69	2000	10	0.05	29	344,000
H ₄	76	2000	10	0.10	63	352,000
H ₅	83	2000	10	0.10	32	200,000
H ₆	90	250	10	0.20	37	114,000
H ₇	97	200	5	0.25	45	112,000
H ₈	104	200	10	0.25	23	50,000
H ₉	111	200	10	0.50	22	23,000

4. The results agree very closely with those of Cole [1938] except that we observed a more rapid rate of disappearance of the hormone.

5. Withdrawal of blood to the extent recorded, which was as much as 10% of the total volume weekly for 4 weeks, had no effect on the course of pregnancy, nor, so far as can be judged from these experiments, did it affect the shape of the curve of the concentration of the hormone in the serum.

The authors desire to thank Drs. R. I. N. Greaves and M. van den Ende for their kind co-operation in drying the bulk of the serum which was collected from the eight ponies.

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PERIODIC UTERINE BLEEDING IN SPAYED RHESUS MONKEYS INJECTED DAILY WITH A CONSTANT THRESHOLD DOSE OF OESTRONE

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WHEN a high dose of oestrone is given daily to a spayed monkey no uterine bleeding occurs until some days after the cessation of the course of treatment. When a sub-threshold dose is given (about 5 μ g. daily) bleeding occurs neither during nor after the period of injections. It was noted in previous communications, however, that periodic uterine bleeding, demarcating what are referred to as threshold cycles, occurred in a spayed rhesus monkey (*M. mulatta*) that was being injected daily with a constant threshold dose of oestrone (10 μ g.) [Zuckerman, 1937*a*, 1938*a*]. This observation is of considerable importance from the point of view of the problem of the endocrine control of the endometrium, and although it represents a critical case, corroborative evidence was therefore desirable. The present note records a further series of experiments in which similar findings were made.

EXPERIMENTAL

Fifteen spayed rhesus monkeys were used. Details of these animals are given in Table I.

The ovaries of each animal were examined by serial histological section to determine whether or not ovulation or marked follicular growth had occurred previous to spaying. Those animals marked 'prepubertal' in Table I had ovaries which were altogether immature; those called 'pubertal' had ovaries in which pubertal growth of follicles had begun; those referred to as 'mature' had ovaries which had at some time definitely ovulated.

All injections were made intramuscularly, the oestrone being dissolved in arachis oil. The occurrence of vaginal bleeding was determined by daily vaginal lavage.

RESULTS

Two of the fifteen animals were given 7.5 μ g. of oestrone daily at the start of the injections.

The first (180) was kept 522 days in the experiment. No threshold cycles were observed, even though the daily dose was repeatedly varied between 7 μ g. and 10 μ g.

The second (213) bled 22 days after the start of the injections, bleeding lasting 3 days. The further history of this animal is discussed below.

The remaining thirteen animals were injected daily, from the start, with 10 μ g. of oestrone. Ten of the thirteen bled periodically. The maximum

Table I. *Details of monkeys used in experiments*

Animal no.	Days under expt.	Body-wt. at start (g.)	Body-wt. at end of expt. (g.)	History previous to present experiment
78	375	2120	3100	Prepubertal. No previous treatment
262	413	2450	3600	Prepubertal. One phase of exptl. oestrogenic stimulation
241	85	2550	2500	Prepubertal. One phase of exptl. oestrogenic stimulation.
220	483	3400	4620	Pubertal. No previous treatment
243	388	2150	2940	Prepubertal. One phase of exptl. oestrogenic stimulation
233	100	4200	4200	Pubertal. No previous treatment
216	112	2500	2940	Prepubertal. No previous treatment
447	310	2600	3330	Prepubertal. No previous treatment
430	310	4020	5000	Pubertal. 8 phases of exptl. oestrogenic stimulation
382	91	6320	6400	Mature. 24 phases of exptl. oestrogenic stimulation after spaying
217	498	3370	4400	Pubertal. No previous treatment
215	333	2730	3320	Prepubertal. No previous treatment
213	179	4000	4740	Mature. No previous treatment
70	289	7100	7130	Mature. 13 phases of exptl. oestrogenic stimulation after spaying
180	522	3960	4800	Pubertal. 4 phases of exptl. oestrogenic stimulation after spaying

experimental period in any one animal was 498 days, during which 14 'threshold cycles' were recorded. The minimum was 85 with 2 threshold cycles. The observations made on these ten animals are analysed below.

One (70) of the three exceptional animals injected with 10 μ g. of oestrone showed no sign of bleeding in the first 52 days of the experiment. Its daily dose of oestrone was then changed to 7.5 μ g., where it was kept till the 101st day. No bleeding occurred during this period or between the 102nd and 106th days, when no injections were given. Fifteen μ g. were administered daily between the 107th and 166th days. Again no bleeding took place, nor was any observed between the 167th and 192nd days when no injections were given. Between the 193rd and 282nd day, 20 μ g. were given daily. No bleeding occurred during this period. No further injections were therefore given. Uterine bleeding began 7 days after their cessation.

No success was thus achieved in attempts to find the threshold level, in

this animal, at which uterine bleeding occurs during the course of oestrone injections.

The second (217) of the three exceptional animals injected with $10\mu\text{g.}$ of oestrone bled on the 41st day of the experiment, bleeding going on for 9 days. It bled again on the 167th day, bleeding continuing for 7 days. These two threshold cycles thus lasted for 41 days and 126 days respectively. No further bleeding was observed by the 250th day. The animal was not injected between the 251st and 260th days. Bleeding began 8 days after the cessation of the treatment.

It appeared, therefore, that in this animal $10\mu\text{g.}$ daily was a supra-threshold level of oestrone at which uterine bleeding was unlikely to occur.

From the 261st day the monkey was therefore given $7.5\mu\text{g.}$ of oestrone daily. It bled on the 302nd day, the bleeding, which lasted 8 days, demarcating a threshold cycle of 42 days. No further bleeding was observed by the 373rd day, and the animal was again given a rest from injection until the 380th day. Bleeding occurred 6 days after the cessation of treatment and lasted 4 days.

From the 380th day $6.5\mu\text{g.}$ of oestrone were given daily. Bleeding occurred on the 416th day, and lasted 11 days, demarcating a threshold cycle of 37 days. No further bleeding was observed by the 498th day and the animal was therefore withdrawn from the experiment.

The level of oestrone stimulation at which threshold cycles occur thus fell progressively in this monkey, the fall being associated with a prolongation of the interval between successive bleedings.

The last of the exceptional animals (215) which was injected daily with $10\mu\text{g.}$ of oestrone experienced three threshold cycles at this level of injection during the first 198 days of the experiment. These cycles were 32, 67 and 99 days, and the periods of bleeding 11, 7 and 6 days long respectively. The second and third cycles were respectively twice and three times as long as the first.

No further bleeding having occurred by the 260th day, injections were discontinued until the 268th day. Uterine bleeding began 3 days after the cessation of treatment and lasted 4 days.

From the 268th day $7.5\mu\text{g.}$ of oestrone were given daily. Bleeding occurred on the 300th day and lasted 5 days, demarcating a threshold cycle of 33 days. The animal was then removed from the experiment.

In all 68 threshold cycles were observed in the animals injected with $10\mu\text{g.}$ daily. The mean length of cycle was 43 ± 2.6 days. This is not significantly higher than the mean length (37 ± 2.1 days) of the 125 normal rhesus cycles recorded by Corner [1923], but statistical analysis shows that it is significantly greater than the mean length of the 1,000 normal sheep cycles (33.5 ± 0.6 days) which were analysed in an earlier publication.

[Zuckerman, 1937*b*]. These 1,000 cycles were compiled from data provided by Hartman [1932] on 708 cycles, by Corner [1923] on 125 cycles and by Zuckerman [1937*b*] on 167 cycles. The reason for this discrepancy is not as yet clear.

The mean length of the periods of uterine bleeding in the threshold cycles was 7 ± 0.1 days.

After threshold cycles had been established, four animals were hypophysectomized through a fronto-temporal approach. Only one of the four survived the first week after the operation. This animal (213) lived for 141 days, during which it experienced one threshold cycle of 49 days on $7.5 \mu\text{g.}$ of oestrone daily. It may be noted that threshold oestrous cycles occur in hypophysectomized rats [Zuckerman, 1938*b*; Bourne & Zuckerman, 1941 *a, b*].

Another spayed monkey (262) in which threshold cycles had been established on $10 \mu\text{g.}$ a day was bilaterally adrenalectomized, the right adrenal being removed a week before the left. The animal was given 2 mg. of desoxycorticosterone acetate daily, in addition to $10 \mu\text{g.}$ of oestrone, after the removal of the first adrenal. It lived 27 days after it had been bilaterally adrenalectomized, but uterine bleeding did not occur during this period.

SUMMARY

Ten of fifteen spayed rhesus monkeys which were injected daily with a constant threshold dose of oestrone experienced periodic uterine bleeding during the course of treatment (which varied from 85 to 498 days). The mean interval between 68 recorded 'inter-menstrual' periods ('artificial threshold cycles') was 43 ± 2.6 days. The mean length of normal menstrual cycles in one control series [Corner, 1923] was not significantly lower (37 ± 2.1 days), although that of a more comprehensive series of 1,000 cycles was (33.5 ± 0.6 days). One of the remaining five animals experienced an artificial threshold cycle after hypophysectomy. The parts played by the pituitary and adrenals in the maintenance of artificial threshold cycles are discussed elsewhere in relation to observations made on rats [Bourne & Zuckerman, 1941*a, b*].

I am greatly indebted to Professor Hugh Cairns and Mr. J. Pennybacker for the help they gave in carrying out the hypophysectomies and adrenalectomies.

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THE INFLUENCE OF THE ADRENALS ON CYCLICAL CHANGES IN THE ACCESSORY REPRODUCTIVE ORGANS OF FEMALE RATS

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UTERINE bleeding occurs in spayed monkeys treated with oestrogens only when the intensity of stimulation falls below a certain threshold level. Thus it takes place not during, but only some days after, the cessation of a course of daily injections of a high dose of oestrone. If the daily dose is of a sub-threshold value, it does not occur at all. On the other hand, it occurs periodically during the course of daily injections of threshold doses of oestrone [Zuckerman, 1937, 1938a, 1941], the period (so far as available observations allow of any definite conclusion on the point) being slightly longer than the normal menstrual period, although of the same order of magnitude. A daily 'threshold' dose of oestrogen thus periodically becomes insufficient for maintaining endometrial growth. The obvious inference is that rhythmical fluctuations occur in the sensitivity of the uterus to oestrogenic stimulation, or that some factor other than the ovaries also exerts an influence on the cyclical changes that take place in the endometrium.

Consideration of what this factor could be led to the working hypothesis that the adrenal cortex may be responsible both for the persistence of uterine cycles in spayed monkeys that are injected daily with a constant threshold dose of oestrone, and for cyclical variations in uterine sensitivity [Long & Zuckerman, 1937; Zuckerman, 1938a]. One suggestion was that cyclical alterations in the activity of the adrenal cortex may lead to cyclical hydration and dehydration of the accessory reproductive organs, and that such changes could determine variations in the responsiveness of the organs to oestrogenic stimulation.

Another possibility is that the adrenal cortex produces a sub-threshold amount of oestrogenic hormone itself, the amount produced varying cyclically, becoming less, or negligible, at times corresponding to uterine bleeding. The amount of effective oestrogen acting on the uterus of a spayed monkey injected daily with 'threshold' doses of oestrogen would thus be the introduced hormone supplemented by the oestrogen produced daily by the adrenals. Either source would by itself be insufficient to maintain the endometrium in a phase of growth, and bleeding would thus occur at times when the adrenal was not secreting any oestrogen.

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A third possibility is that the adrenal cortex cyclically produces, at times of uterine bleeding, androgenic and/or progestational hormone, and that this endogenous hormone neutralizes the effect of the introduced oestrogen at those times. Alternatively, such hormone may be produced continuously, but in amounts which vary cyclically.

Owing to the difficulty of investigating the problem on monkeys an attempt was made to discover whether similar threshold cycles occur in spayed rats. When the problem came to be investigated, it was found that some information bearing on the question had already been published. Kostitch & Télébakovitch [1929] had observed that a vaginal cycle of epithelial changes occurs in spayed as well as in normal mice, a preponderance of mucus, leucocytes and nucleated epithelial cells following each other in regular order. An observation more to the point was that of Del Castillo & Calatroni [1930], who found that oestrus recurred periodically in a group of spayed rats which were injected daily with a constant low dose of oestrone.

This observation was confirmed in a preliminary set of experiments carried out on 23 spayed rats of an impure hooded strain [Zuckerman, 1938*b*]. The individual variation in the level of oestrogenic stimulation at which periodic oestrus occurred (demarcating 'artificial oestrous cycles') was from 5 to 15 I.U. of oestrone (given subcutaneously in oil solution at a concentration of 10 $\mu\text{g.}$ per ml.), the mean being 8.9 I.U. If too much oestrone was given, continuous oestrus was produced. If too little, phases of prolonged anoestrus occurred. The modal and maximum duration of oestrus in normal rats are 27 and 81 hours respectively [Long & Evans, 1922]. Since the animals used in these experiments were smeared only once daily, cycles in which oestrus lasted for 4 days or more were taken to indicate that too much oestrone was being given, and were left out of the calculations. Cycles of 10 days or of longer duration were regarded as phases of anoestrus, and were also excluded. Such cycles occurred in only 6% of Long & Evans's series of 1999 observations, and the chance of being in error in leaving them out was very slight.

A total of 203 successive 'artificial oestrous cycles' was recorded in the 23 rats investigated in this preliminary study. The mean cycle-length was 5.3 days. The mean duration of the normal oestrous cycle of the rat is 5.4 days [Long & Evans]. As in the normal animal, too, the most frequent cycle-lengths were 4, 5 and 6 days.

Further information bearing on the question has since been published by Di Paola [1939]. This worker injected 4 groups of 5 spayed rats each with 0.5, 1.0, 5.0 and 20.0 I.U. of oestradiol benzoate daily for 90 days respectively. Eight control rats were injected with oil alone.

Almost continuous oestrus was observed in the rats given 5 and 20 I.U.

daily. Those given 1 I.U. came into oestrus after 2 to 3 days, and in each there was an average of 9 oestrous phases lasting from 3 to 8 days, during the 90 days of the experiment. Two of the animals given 0.5 I.U. daily had two oestrous periods in the 90 days, 4 had 4, and 1 had none.

The first step in the present investigation was to obtain further information about the occurrence of these threshold artificial oestrous cycles. The second was to discover whether or not these cycles continue in the absence of the pituitary or adrenal glands, or in the absence of both.

MATERIAL AND METHODS

Animals

154 young sexually-mature female white rats, all raised in this laboratory from the same inbred pure Glaxo (Wistar) strain, and weighing at autopsy between 148 and 196 g., were used. Preliminary observations on the duration of the normal oestrous cycle were made on 44, 15 of which were then adrenalectomized in order to determine the daily amount of desoxycorticosterone acetate necessary to maintain the animals in good health and with normal oestrous cycles.

All but 20 of all the animals were subsequently spayed. Attempts were made to establish threshold artificial oestrous cycles in 120. Seventeen of the animals in which they were established were subsequently hypophysectomized and 27 adrenalectomized. Six of the hypophysectomized rats were later adrenalectomized.

Care of animals

The animals were all kept on the same constant diet of rat-cake and greens, supplemented twice weekly with brown bread, milk, liver or meat. The animal-room was kept at a constant temperature of 65° F.

Injections

All vaginal examinations and injections were carried out at approximately the same time each day. The hormones were administered subcutaneously in nut-oil solution. Vaginal changes were followed by means of the lavage method.

Phases of oestrus occurred periodically in animals given daily doses of oestrone varying between 3.75 and 10.0 I.U. The level of oestrone stimulation at which they occurred was significantly lower when the rats were given priming doses of oestrone at the time they were spayed. Thus the average daily dose on which animals given a priming dose of 250 I.U. of oestrone periodically came into oestrus was 4.107 ± 0.115 I.U.; without a preliminary priming dose it was 6.736 ± 0.131 I.U. Priming doses were given the day after spaying, and the daily injections of threshold doses of

oestrone were usually begun four days later. When no priming dose was given the daily injections were begun towards the end of the first week after spaying.

Criteria for defining threshold artificial oestrous cycles

As noted above, if a spayed rat is given too much oestrone daily, it goes into continuous oestrus. If too little is given, phases of prolonged anoestrus set in. In order to find the critical level at which phases of oestrus occurred periodically, it was thus necessary to alter the daily dose from time to time. Interoestral periods during which it became necessary, for one or other of these reasons, to alter the daily dose of oestrone have not been regarded as 'threshold artificial oestrous cycles'. These are strictly defined as interoestral periods, occurring during the administration of a constant daily dose of oestrone, that conform to the criteria which emerge from the following considerations.

The mean length of the oestrous cycle in the normal rat is in the region of 5 days (5.4 days) [Long & Evans, 1922]. It is possible, however, judging by Long & Evans's data, for a rat to go through all the phases of an oestrous cycle in a period of only 48 hours. Threshold cycles made up of a single day of oestrus and one of dioestrus were therefore included in our calculations.

The longest period of oestrus observed by Long & Evans in their series of 1999 cycles was $3\frac{1}{2}$ days. If oestrus continued for more than 3 days in our experimental animals, as happened occasionally (without any change in the daily dose of oestrone) in series with shorter phases, the cycles concerned were left out of the calculations. If necessary the daily dose was then reduced.

If a period of anoestrus lasting as much as 10 days occurred in a spayed animal, it was taken that too little oestrone was being given daily, and in such cases the daily dose was increased. Consequently, threshold cycles of 10 days or longer were excluded from consideration in the final results.

Operations

All animals were spayed by the usual dorsal approach.

Hypophysectomies were carried out by the retropharyngeal approach. The hypophysectomized rats were weighed daily. All showed a steady decline in weight from the beginning to the end of the experiment. At autopsy all soft tissue in the hypophyseal region was removed and serially sectioned. No glandular tissue was found.

Adrenalectomies were performed, under ether anaesthesia, by the usual dorsal approach. The adrenals were removed in their fatty capsules. In addition to the constant threshold doses of oestrone, the adrenalectomized

animals were injected daily with 1 mg. of desoxycorticosterone acetate in oil. The amount given was determined by control experiments (see below) which indicated the daily dose of hormone necessary to maintain normal oestrous cycles in rats that had been adrenalectomized but which retained their ovaries. All the adrenalectomized animals died at varying periods after the discontinuation of the desoxycorticosterone treatment at the end of each experiment. Each adrenalectomized animal was carefully examined at autopsy, and where necessary by histological section of the adrenal beds, for any accessory adrenal tissue. None was found.

In addition to the normal diet both hypophysectomized and adrenalectomized animals were given bread, milk and wheat-germ daily.

Methods of statistical analysis

All our data were analysed statistically by methods outlined by Fisher [1932]. Means were compared by means of the 't' test. Differences were regarded as being significant statistically when 'P' was 0.02 or less, i.e. when there was less than one chance in 50 that a difference between means would attain the observed magnitude if the samples were random ones drawn from the same population.

EXPERIMENTAL

Duration of oestrous cycle in normal female rats of strain used in this study

Since interoestral periods lasting for 10 days or more were chosen arbitrarily as implying a failure to find the correct threshold level at which periodic oestrus occurs, it became necessary to exclude, from the normal control data collected for purposes of comparison, oestrous cycles which were longer than 9 days, even though their occasional occurrence is a normal phenomenon. (They occurred in 17, or 10.76% of 158 cycles in normal rats of the strain used in this study. In Long & Evans's series of 1999 observations they occurred in 9% of cases.)

One hundred and fifty eight normal cycles were observed in 44 animals. The minimum number of cycles observed in any one animal was 1, the maximum 9. Excluding 17 exceptional cycles which were longer than 9 days, the mean length of cycle in the remaining 141 cases was 5.227 ± 0.127 days. In Long & Evans's series most cycles were of 4, 5 and 6 days, in that order of frequency. In ours the order was 5, 4, 6. In none of our 158 normal cycles was oestrus prolonged for more than 3 days.

Threshold cycles in spayed rats

Attempts to establish threshold cycles were made in 120 rats. Thirty-three or 7.40% of a grand total of 446 cycles were omitted from the final

calculations because they were longer than 9 days. Periods of extended oestrus (also discarded) occurred 59 times (13·22%) in the whole series of 446 cycles. Three hundred and fifty four cycles, or 79·37% of the grand total, agreed with the criteria defined above, the minimum number of cycles followed in any single animal being 1, and the maximum 6.

The mean length of 354 threshold cycles was $4·811 \pm 0·108$ days. Statistical analysis shows that the difference between this mean and the mean of the control series of normal oestrous cycles is not significant (diff. = 0·416, $n = 493$, $t = 2·206$, P lies between 0·05 and 0·02).

On the other hand, analysis shows that the mean length of threshold cycle in the present series of animals is significantly less than the mean figure in the preliminary series that was studied (diff. = 0·514, $n = 557$, $t = 2·896$, $P = \text{less than } 0·01$) [Zuckerman, 1938*b*]. This difference is probably explained by the fact that the previous set of observations were made on an impure strain of hooded rats, whereas the present observations were made on a pure strain of Glaxo (Wistar) rats.

In the case of the monkey, the periodic occurrence of uterine bleeding implies that the threshold daily dose of oestrone becomes too low at such times to maintain the endometrium in a phase of growth. In the case of the rat, the occurrence of cyclical oestrus implies that the effect of the constant daily threshold dose of oestrone becomes greater at such times, for any of the reasons detailed on pp. 268-9. The only alternative to the possibilities that have been suggested is the very unlikely hypothesis that a threshold amount of oestrone injected daily has a cumulative effect in the body, the peak of action occurring every five days in a rhythm corresponding to the normal oestrous cycle (the corresponding rhythm in the monkey being approximately that of the menstrual cycle). If such an explanation were resorted to, it immediately raises the problem of the species difference in periodicity of threshold cycles. This hypothesis also implies that in the case of the rat, the concentration of the injected oestrone rises in the body until it exceeds a threshold at which the vaginal mucosa responds, and that the concentration then falls as a result of the utilization of the hormone, to rise again in another 5 days. This formulation of the problem merely restates the issue in terms of the question, how do bodily factors governing the storage, destruction and excretion of the hormone in the rat operate in a cycle corresponding to the normal 5-day oestrous cycle?

Whatever be the explanation, the fact that oestrus occurs in a cycle of approximately 5 days in rats which are given a threshold daily dose of oestrone indicates that some factor other than the ovaries must be concerned in the control of the cyclical change which takes place normally in the accessory reproductive organs.

Threshold cycles in hypophysectomized rats

It is generally agreed that the anterior lobe of the pituitary does not exert a direct hormonal influence on the accessory reproductive organs, but that it exerts its effects indirectly by way of the ovaries. Thus oestrous cycles cease in hypophysectomized rats. On the other hand, it is conceivable that the anterior lobe has a direct or indirect influence on the general metabolism, or on the water metabolism (by way of the adrenal glands) of the accessory organs. It was consequently of interest to discover what effect hypophysectomy has on the occurrence of threshold artificial oestrous cycles.

In a previous set of experiments [Zuckerman, 1938c] carried out on 8 spayed rats it was found that threshold cycles continued after removal of the pituitary. The cycle did not alter significantly in length, but the threshold level at which it showed itself was consistently lower than in control animals which had only been spayed (a mean of 4 I.U. of oestrone daily as compared with the mean of 8.9 I.U.). When the injections were continued at the same level as before, 5 of 8 other rats in which artificial cycles had previously been established went into, and remained in oestrus during the survival period following the operation.

In the present study an attempt was made to establish threshold cycles in 17 spayed Glaxo rats after hypophysectomy. The attempt was successful in every case, and a total of 98 cycles, or 89.09% of a grand total of 110 interoestral periods agreed with the criteria which were decided upon as characterizing a threshold artificial cycle. The average number of days each hypophysectomized animal was kept under observation was 42, the range being 15 to 63. The minimum number of cycles recorded in any one animal was 2, and the maximum 10. There were only 4 cycles longer than 9 days (3.64% of the whole series), and 8 cycles in which oestrus continued for more than 3 days (7.28% of the whole series). These were excluded from the final calculations.

The mean length of threshold cycle after hypophysectomy was 4.694 ± 0.177 days. Statistical analysis showed that this mean is not significantly less than the mean for threshold cycles in rats that had only been spayed (diff. = 0.117, $n = 450$, $t = 0.520$, $P = 0.6$). The mean duration of threshold cycle in the hypophysectomized rats proved, however, to be significantly less than the mean length of the normal cycles in our control series (diff. = 0.533, $n = 237$, $t = 2.516$, P lies between 0.02 and 0.01).

In the preliminary set of experiments the mean daily dose of oestrone on which spayed hypophysectomized animals underwent threshold cycles was lower than the dose on which animals that had only been spayed experienced cycles. In the present set of experiments the threshold for

cycles was the same before and after hypophysectomy in 10 of the 17 animals. It was higher in 3 and lower in 4. The mean for the whole series was the same before as after removal of the pituitary (4.265 I.U. daily).

Threshold cycles in adrenalectomized rats

In every mammalian species that has yet been tested complete removal of the adrenal glands leads to cessation of the oestrous cycle and death [see Grollman, 1936]. Bilateral adrenalectomy in the rat is occasionally not followed by death, presumably because of the existence of accessory adrenal tissue, and in such cases oestrous cycles continue. Similarly, if adrenalectomized rats can be kept alive on salt, the cycle may proceed normally [Kutz, McKeown & Selye, 1934]. Completely adrenalectomized animals maintained in good health by treatment with extracts of the adrenal cortex, or with desoxycorticosterone, also have normal reproductive functions.

In order to find out whether threshold oestrous cycles occur in spayed rats after adrenalectomy, it was first necessary to determine what amounts of desoxycorticosterone acetate are required to maintain adrenalectomized unspayed rats in a healthy condition in which oestrous cycles proceed normally.

Fifteen normal rats whose oestrous cycles had been followed for varying periods were accordingly adrenalectomized and given either 0.5, 1.0, or 2.0 mg. of desoxycorticosterone acetate. Normal oestrous cycles continued in all the animals. The minimum number followed in any one animal was 2, and the maximum 5. The mean duration of 50 cycles was 5.700 ± 0.259 days. Analysis showed that this mean is not significantly greater than the mean of the 141 normal cycles in the control series (diff. = 0.473, $n = 189$, $t = 1.801$, P lies between 0.1 and 0.05).

Having established the amount of desoxycorticosterone acetate necessary to maintain oestrous cycles in an adrenalectomized but unspayed rat, 27 spayed animals in whom threshold cycles had been established were adrenalectomized and given, in addition to their daily threshold dose of oestrone, a daily maintenance dose of 1 mg. of desoxycorticosterone acetate. The average period these animals were kept under observation was 42 days, the range being 8 to 86.

In only 4 of the 27 animals was it possible to establish a series of more than 2 threshold cycles (a total of 19 cycles in all). The remainder either failed to come into oestrus at all, or experienced prolonged periods of dioestrus or, when the daily dose of oestrone was raised, of oestrus. Occasionally an interoestral period which agreed with the criteria defined above was observed. There were 21 periods of extended dioestrus in which attempts to bring the animals into oestrus by altering the daily dose of

oestrone within threshold range failed; 16 threshold cycles in which the period of oestrus was prolonged for more than 3 days; and 17 threshold cycles which were longer than 9 days in duration.

In the whole series of 99 there were only 45 interoestral periods (or 45.45% of the total) which agreed with the criteria by which a threshold cycle has been defined. These have been analysed statistically even though, as noted above, the majority represent occasional cycles occurring in series with periods of extended oestrus or extended dioestrus.

The mean duration of these 45 cycles was 5.400 ± 0.303 days. Analysis shows that this mean is not significantly longer than that of control threshold cycles in animals which had only been spayed (diff. = 0.589, $n = 397$, $t = 1.835$, P lies between 0.1 and 0.5). It also showed that the threshold cycle in adrenalectomized rats was not significantly shorter than the 'normal' oestrous cycle in adrenalectomized unsplayed animals (diff. = 0.300, $n = 93$, $t = 0.756$, P lies between 0.05 and 0.04).

Twelve of the 27 spayed adrenalectomized animals had undergone threshold cycles before the removal of their adrenals. The level of oestrogenic stimulation at which occasional cycles appeared after adrenalectomy remained the same in only 4. In the remaining 8 the daily dose of oestrone had to be raised. In general it seemed that more oestrone is necessary to stimulate oestrus in an adrenalectomized rat than in one possessing its adrenals.

This observation does not agree with other published information. According to Schwabe & Emery [1939] total adrenalectomy does not affect the minimum dose of 'theelin' required to produce oestrus in a spayed rat. The discrepancy in this case may be due to the fact that theelin, an extract, was used in their experiments and oestrone in ours. Di Paola's [1939] observations led him to the view that adrenalectomized spayed rats are more sensitive than spayed rats to oestradiol benzoate, less being required to bring them into oestrus. His report suggests that after adrenalectomy animals injected daily with 1 I.U. of the hormone went into continuous oestrus and no longer experienced threshold cycles, while animals which on 0.5 I.U. had only occasionally, or never, come into oestrus, showed an oestrous reaction. In no case apparently did he observe threshold cycles which accord with the criteria defined in this paper. In this case the discrepancy may be due to the fact that an ester of oestradiol, which is more slowly utilized than oestrone, was used.

Our own findings show that it is difficult to establish threshold cycles in spayed rats after adrenalectomy, in spite of the administration of an amount of desoxycorticosterone that maintains reproductive function in unsplayed rats, and agree with the results of a preliminary series of experiments [Zuckerman, 1938c]. In this preliminary study 7 spayed rats in

which a series of successive threshold cycles had been established were adrenalectomized and given a maintenance dose of cortical hormone daily, oestrone being administered as before. Cycles continued in only 3 of the animals, but were more prolonged than normal. Repeated attempts to establish cycles in the others by varying the dose of hormone given daily were unsuccessful.

It follows from all these experiments that the occurrence of threshold artificial oestrous cycles in spayed rats is in some way dependent on the normal functioning of the adrenal glands.

Threshold cycles in adrenalectomized-hypophysectomized rats

In the preliminary series of experiments [Zuckerman, 1938c], 3 hypophysectomized animals in which cycles had previously been established were adrenalectomized and then injected daily, like the animals in the previous set of experiments, with a maintenance dose of cortical hormone and with the usual threshold dose of oestrone. Attempts to continue the cycles were unsuccessful. Anoestrus or phases of prolonged oestrus made up the survival period after the operation (up to 24 days), in spite of varying the daily dose of oestrone.

Six further animals were treated in the same way in the present set of experiments. The results were identical. The first rat, in which regular threshold cycles had been established after hypophysectomy, failed to come into oestrus for 10 days after the removal of its adrenals. The daily dose of oestrone was then raised from 5 to 6.25 I.U. Oestrus occurred on the 7th day after this change and continued for 2 days. It reappeared after an interval of 1 day, and the animal then went into dioestrus for a further period of 6 days. The daily dose was accordingly raised to 7.5 I.U. Oestrus occurred 6 days later, and lasted 1 day. It was succeeded by 5 days of dioestrus, which in turn were followed by 7 of oestrus. The animal was found dead on the 43rd day after the removal of its adrenals.

The history of a second animal in this series was very similar. In the remaining 4 animals attempts to establish cycles failed completely.

DISCUSSION

Sufficient information is now available to make it clear that when kept at a critical and constant threshold level of oestrogenic stimulation, spayed rats come into oestrus in periods that correspond to the normal oestrous cycle. Since the amount of oestrone given daily during the elicitation of these threshold artificial oestrous cycles is constant, it is obvious that under these conditions some internal bodily factor regulates the periodic response of the necessary reproductive organs. The ovaries, controlled by the anterior lobe of the pituitary, are generally regarded as a fully respon-

sible for the cyclical changes which take place in the accessory organs during the normal cycle. They cannot, however, be responsible for the cyclical changes which occur in spayed animals, and it would therefore seem that normally some extra-gonadal factor also exercises a periodic effect upon the mucosa of the accessory reproductive organs.

Since threshold artificial cycles continue in spayed rats after hypophysectomy, the pituitary can neither be involved directly in this extra-gonadal control of the accessory organs, nor indirectly through the general endocrine and metabolic disturbances which occur after its removal alone. Involution of the adrenals is one of these disturbances. The adrenals, however, remain sufficiently functional for hypophysectomized animals to live in good health for relatively long periods. It is thus conceivable that they constitute the factor responsible for the maintenance of threshold artificial cycles in spayed animals both before and after removal of the pituitary.

This conclusion is supported by the fact that when the adrenals are removed, the experimental elicitation of threshold cycles becomes very difficult, although they may still continue in some animals. The fact that they do, even when the deficiency effects of the adrenalectomy are corrected by a constant daily dose of desoxycorticosterone acetate, makes the problem difficult to interpret. From one point of view these exceptional cases could be regarded as critical, for the continuation of cycles in even a few adrenalectomized animals suggests at first sight that the influence of the adrenals cannot be specific in any of the three ways defined in the introduction to this paper (pp. 268-9).

On the other hand, it must be remembered that a succession of threshold artificial cycles could be elicited in only 4 of our 27 adrenalectomized animals. In spite of the fact that no accessory adrenal tissue was discovered at autopsy or by microscopic examination of the adrenal beds, it is conceivable that such tissue was actually present in these 4 rats. Whether or not this be the explanation for the exceptional cases, what is more significant than the fact of their occurrence is the observation that threshold cycles could not be elicited in the majority of our adrenalectomized animals. Such being the case, it is reasonable to suppose that the adrenals either have a specific influence on the accessory reproductive organs, or that the changed behaviour of these organs after removal of the adrenals is merely one of many 'non-specific' symptoms of adrenal insufficiency.

In so far as our adrenalectomized animals were provided daily with ample quantities of desoxycorticosterone acetate to preserve them in good health, it is difficult to accept this latter view. They showed no obvious sign of insufficiency, nor any toxic symptoms resulting from their daily

injections of oestrone—although oestrogens are known to be toxic to adrenalectomized mice and rats [Cramer & Horning, 1939; Selye & Masson, 1939]. This may have been due to the very small amounts of oestrone they were given. Alternatively, it may have been due to the fact that they had been given oestrone before their adrenals were removed, for Cramer & Horning point out that mice which are pre-treated with oestrogens before adrenalectomy are able to resist the toxic effects of injected oestrogen, a finding which Selye & Masson regard as indicating an adaptation similar to the 'stage of resistance' in the 'alarm reaction' defined earlier by Selye [1937]. In any event, preliminary observations which we have made show that oestrogens are not toxic in adrenalectomized animals that are kept in good health by means of desoxycorticosterone.

The usual failure to elicit threshold artificial cycles in spayed rats after adrenalectomy can hardly be taken, therefore, to represent a generalized and non-specific effect of adrenal insufficiency. As already seen, however, threshold cycles are even more difficult to elicit in hypophysectomized-adrenalectomized animals than in animals which are only adrenalectomized. This fact suggests that apart from what seems to be a specific effect of the adrenals on the accessory organs, the occurrence of threshold cycles is influenced by the effects which the pituitary has upon the endocrine system generally—in spite of the fact that hypophysectomy alone does not influence the threshold cycle. This conclusion can, however, only be stated tentatively, in so far as the number of cases on which it is based is small.

Observations reported in the following paper show that the adrenal glands of spayed rats fluctuate in size during threshold cycles. Since it is likely that the inhibition of the cycle after adrenalectomy is a specific effect, the question which arises at this point is whether the adrenals influence the accessory reproductive organs directly, or whether they do so through the medium of some other endocrine organ.

Consideration of the available evidence suggests that they do not exert their influence indirectly. Thus it is plain that their effects are mediated neither through the ovaries nor through the pituitary—since threshold cycles continue after the removal of both. One of the remaining possibilities is that they exert their effects on the reproductive system through the thyroid. The evidence, however, is against this thesis. The thyroid, although not essential for reproduction in animals, undoubtedly has some effect on reproductive function [Reiss & Pereny, 1928; Leonard, 1936; Zalesky & Wells, 1937; Benoit, 1937]. On the other hand, available evidence does not suggest that the adrenals and thyroid interact specifically or intimately [Grollman, 1936]. Many observers have reported thyroid over-activity after adrenalectomy, and others (e.g. Deane &

Uyldert, 1937] thyroid inactivity. According to Grollman, temporary over-activity is probably a reflex effect of the trauma of the operation, and not due to dysfunction of the adrenals. Under-activity, according to Dessau & Uyldert, is due, not to diminished responsiveness of the thyroid to thyrotrophic hormone, but to diminished secretion of that hormone by the pituitary. Whatever be the true facts and correct explanation, it is obvious that there is no evidence which suggests any specific connexion between the thyroid and adrenals on the one hand, and between the thyroid and accessory reproductive organs on the other.

The last obvious possibility of indirect hormonal action is that the adrenals exercise their influence on the accessory reproductive organs by way of the thymus. The activity of the thymus is linked in some way with that of the reproductive system. The gland normally decreases in size after puberty, while gonadectomy in young animals leads to thymic involution [Nelson, 1939]. Furthermore, the administration of oestrogen or androgen leads to a decrease in the size of the organ [Inay & Thompson, 1938; Selye & Masson, 1939; Levic, Uyldert, & Dingemans, 1939; Cramer & Horning, 1939]. There is also a definite relation between the thymus and the adrenal glands. Thus it is known that excessive muscular exercise, or the administration of noxious stimuli, leads to enlargement of the adrenals and atrophy of the thymus, and that atrophy of the thymus does not occur under these conditions after removal of the adrenals [Leblond & Segal, 1938]. The thymus may in fact increase in size after adrenalectomy [Cramer & Horning, 1939]. The only agents known to cause thymic involution in rats, in the absence of the adrenals, are adrenal cortical hormone, oestrogenic hormone, and androgenic hormone [Selye & Masson, 1939]. According to Cramer & Horning, however, thymic involution either does not result from oestrogenic treatment, or is very diminished, after adrenalectomy. This conclusion is not accepted by Selye & Masson, but neither they, nor Cramer & Horning, provide the quantitative data which allow any decision on this point.

All these observations suggest the existence of some physiological interaction between the adrenals, the thymus and the sex hormones. There is no evidence, however, that the thymus has any influence on the accessory reproductive organs themselves. In the circumstances, it is idle to speculate on the possibility that the adrenals exert their periodic effects on these organs indirectly by way of this gland.

By exclusion, and in the absence of contrary evidence, it must therefore be tentatively concluded that the influence of the adrenals on the cyclical changes which occur in the accessory reproductive organs after spaying is a direct one. The paper which follows accordingly analyses in detail the changes that take place in the adrenal glands during the

necessary to stimulate oestrus in adrenalectomized rats than in rats possessing their adrenals.

6. Six spayed-hypophysectomized rats were adrenalectomized. Attempts to establish artificial threshold cycles in these animals failed completely.

7. Consideration of the evidence suggests that the elicitation of artificial threshold oestrous cycles is dependent on the presence of the adrenals, and that their influence on the cyclical changes which occur in the accessory reproductive organs is a direct one.

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CHANGES IN THE ADRENALS IN RELATION TO THE NORMAL AND ARTIFICIAL THRESHOLD OESTROUS CYCLE IN THE RAT

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THE knowledge that the ovaries are the organs mainly responsible, or as is generally believed, entirely responsible for the control of the cyclical changes which occur in the accessory reproductive organs is based upon three sets of observations: (i) those which show that ovariectomy is followed by the apparent cessation of all cyclical activity in the accessory organs; (ii) those which show that the phases of the ovarian cycle are correlated with the cycle of changes that take place in the accessory reproductive organs; and (iii) those which show that the latter changes can be reproduced in spayed animals by the administration of ovarian hormones.

The view that the adrenal cortex is also concerned in the control of the cyclical changes to which the accessory reproductive organs are subject is so far based entirely on the fact that threshold artificial oestrous cycles [Bourne & Zuckerman, 1941] cannot, as a rule, be elicited after adrenalectomy. Further evidence that the adrenal cortex is concerned is provided by observations, analysed below, which show that the adrenal cortex undergoes cyclical changes in spayed animals in period with the phases of the threshold artificial oestrous cycle.

The fact that the adrenal cortex undergoes such changes in the normal oestrous cycle is already known. Stilling [1898] noted that the adrenals of frogs are enlarged at the time of ovulation. Kolmer [1918], again, observed that the adrenal of the mole enlarges during the mating season, while Riddle [1923] noted adrenal enlargement in pigeons at the time of ovulation. Enlargement of the cortex during oestrus in the mouse was observed by Masui & Tamura in 1926 (although neither Howard-Miller [1927] nor Deanesly [1928] were able to find that the histology of the cortex varies during the cycle). In a detailed study of the rat Andersen & Kennedy [1932] were able to show that the adrenal cortex, but not the adrenal medulla, enlarges significantly during oestrus. According to these workers the cells of the zona fasciculata and of the cell nests adjacent to the medulla enlarge at that time and contain an increased amount of lipoid which stains pale pink with Sharlach R. They also point out that although the evidence indicates a close relation between the adrenal cortex and

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oestrous changes in the reproductive organs, there is no evidence which shows that this represents a specific causal relationship. Grollman [1936] in reviewing the evidence suggests, however, that since 'the reproductive cycle is accompanied by such profound changes in the general activity of the body as a whole, one need not attribute any specific importance to the changes occurring in the adrenal cortex. Such changes may be looked upon merely as a reflection of increased body activity requiring among other things an hyperfunction of the adrenal cortex.' The basis for this far-reaching opinion is not indicated.

Changes in the adrenals in relation to the reproductive cycle have also been reported in the Thirteen-Lined Ground-Squirrel (*Citellus tridecemlineatus*) [Zalesky, 1934]. Cortical hypertrophy occurs in both the male and female of the species during the breeding season, or during anoestrus as a result of stimulation of the gonads by means of gonadotrophic hormone. 'Histologically, this cortical hypertrophy is associated mainly with the expansion of the reticular zone and the differentiation, within it, of a characteristic, highly developed outer sub-zone termed "reticularis A".' Changes in cell type in the adrenal glands also occur during the oestrous cycle of ewes [Nahm & McKenzie, 1937], but it is not reported whether the glands fluctuate in total size during the cycle.

In a preliminary study [Zuckerman, Bourne & Lewes, 1938] it was established that changes similar to those that occur in the normal oestrous cycle take place in the adrenal glands during threshold artificial oestrous cycles. In this study threshold artificial cycles were established in 38 spayed rats. The adrenal glands were removed from half of these animals during oestrus, and from the other half during dioestrus. It was found that the oestrous adrenals were significantly larger than those removed in dioestrus. These preliminary observations suggest strongly that the cyclical changes which occur in the adrenal glands of the normal rat are not merely a reflection of the rhythmical changes which take place in the ovaries.

The experimental observations reported below were designed to provide a test of these preliminary observations, and to analyse the adrenal changes in closer detail.

MATERIAL AND METHODS

Animals

The animals used in the present study were the same 154 inbred pure Glaxo rats which formed the basis of our study of the threshold artificial cycle [Bourne & Zuckerman, 1941]. Of these animals, details of which have already been given, 3 groups of 20 each were used for making observations on changes in the adrenals. The first was used for making observa-

tions on the size of the adrenal in normal oestrus (10 animals) and normal dioestrus (10 animals). The second was used for making observations on the adrenal gland in the oestrous (10 animals) and dioestrous phases (10 animals) of threshold artificial oestrous cycles. The third group was used for making observations on the size of the adrenals 15 days after spaying when large doses (50 μ g.) of oestrone were given daily during the post-operative period (10 animals) and on adrenal-size 15 days after spaying when no treatment was given (10 animals).

Measurement of adrenals

All adrenals were removed at the same relative times during oestrous and dioestrous phases (either on the first day of oestrus or on the second day of dioestrus).

After their removal the adrenals were carefully freed from surrounding fatty tissue and rapidly weighed on a torsion balance. They were then put in Bouin's fluid for 24 hours, and after fixation were serially sectioned at 10 μ and stained with haemalum and eosin.

The outlines of every 10th section were drawn with the help of a projectoscope at a linear magnification of 22 diameters, the boundary line between medulla and cortex being indicated. The areas of medulla and cortex in each section were then measured by means of a planimeter. The volumes of the cortex and of the medulla were estimated from the formula

$$V = \frac{\Sigma \text{ planimeter readings} \times 100\mu}{22^2}.$$

The separate weights of the cortex and medulla in each gland were estimated, on the assumption that the specific gravities of the two are not significantly different, by fractionating the total weight of each gland according to their volumes.

It may be noted that similar methods for determining adrenal volumes have been used by previous workers. An identical procedure was followed by Zalesky [1934]. Andersen & Kennedy [1932] cut their sections at 5 μ and estimated adrenal volumes from measurements of the areas of every 10th section. Deanesly [1931], before adopting an even more simplified technique than our own for estimating relative changes in adrenal volume, measured every other section. All these methods have proved adequate to demonstrate variations which occur in the size of the adrenals.

The separate volumes of the 3 cortical zones in each adrenal gland (zona glomerulosa, zona fasciculata and zona reticularis) were determined in the following way. That section of each adrenal in which the maximum amount of medulla was present was drawn by means of a projectoscope at a magnification (of 44.7 linear diameters) at which the 3 cortical zones

were clearly demarcated (the exact line of demarcation between fasciculata and reticularis was sometimes indistinct). The areas of these zones were then measured by means of a planimeter. The volumes of the separate zones were then estimated by means of the formula

$$\begin{aligned}\text{volume of sphere} &= \frac{4}{3} \pi r^3 \\ &= \frac{4}{3\sqrt{\pi}} a^{\frac{3}{2}},\end{aligned}$$

where a is the area of an equatorial section of a sphere.

The volumes of medulla together with the zona reticularis, the medulla together with the zona reticularis and the zona fasciculata, and the medulla with all the three zones of the cortex, were separately calculated by means of this formula. Subtraction of each volume from the other gave an estimate of the volume of each zone.

This method assumes that the adrenal and its zones can be regarded as a series of concentric spheres. This assumption is, of course, incorrect. An estimate of the error was provided by comparing the calculated volumes for the whole adrenal and medulla with the observed volumes as determined by measurement of the serial sections. Discrepancies were in a few cases large, but nevertheless, as shown in a later section, the method did make it possible to indicate changes which occur in the size of the three zones but which could not be measured directly.

Methods of statistical analysis

All our data were analysed statistically by methods outlined by Fisher [1932]. Means were compared by the t test. Differences were regarded as being significant statistically when P was 0.02 or less, i.e. when there was less than one chance in 50 that a difference between means would attain the observed magnitude if the samples were random ones drawn from the same population.

EXPERIMENTAL

Body-weight

At the beginning of the experiment all the animals weighed about 140 g. They grew at different rates, presumably and partly at least, as a result of the different treatments received, and at autopsy weighed between 148 and 196 g.

Body-weight did not differ significantly in the oestrous and non-oestrous animals of each experimental group (Table I). The greatest difference (8.6 g.) in any one group is between the mean body-weight of the spayed animals injected with a large dose of oestrone daily for 14 days after

spaying and that of the ten spayed animals which were given no treatment and autopsied 15 days after removal of their ovaries. The difference is not significant statistically ($n = 18$, $t = 1.397$, $P = \text{almost } 0.2$, t being 1.330 when P is 0.2).

It will be noticed, however, that although not significantly different, the mean weight for the oestrous animals in each group is less than the mean weight for the dioestrous or anoestrous animals. This agrees with the now widely recognized fact that removal of the ovaries and of the influence of the ovarian secretions leads to an increase in body-weight in rats [e.g. Slonaker, 1930; Freudenberger & Billeter, 1935; Blumenfeld, 1939]. Further evidence that spaying has this effect is indicated by an analysis of the body-weights of our animals, which were all gonadectomized after sexual maturity had been reached.

Table I. *Mean body-weight (in g.) of animals used in this study*

Group 1		Group 2		Group 3	
Normal cycles		Artificial threshold cycles		Animals in constant oestrus or anoestrus	
Oestrus	Dioestrus	Oestrus	Dioestrus	Const. oestrus	Const. anoestrus
158.7 \pm 2.8	160.9 \pm 2.1	177.0 \pm 3.9	178.4 \pm 5.0	161.9 \pm 3.8	170.5 \pm 4.8

All the castrated animals had a greater body-weight than the controls. Thus the body-weight of the artificial-oestrous group is significantly greater than that of the normal-oestrous group (diff. = 18.3 g., $n = 18$, $t = 3.81$, $P < 0.01$). The body-weight of the artificial-dioestrous group is also significantly greater than that of the normal-dioestrous group (diff. = 17.5 g., $n = 18$, $t = 3.24$, $P < 0.01$).

On the other hand the mean body-weight of the animals of the third group which were kept in constant oestrus for 14 days is not significantly greater than those of the normal-oestrous and normal-dioestrous animals. Furthermore, it is significantly less than that of the artificial-oestrous group and that of the artificial-dioestrous group (diff. = 15.1 g., $n = 18$, $t = 2.757$, P lies between 0.02 and 0.01—being 0.01 when $t = 2.878$; diff. = 16.5 g., $n = 18$, $t = 2.625$, P being between 0.02 and 0.01, being 0.02 when $t = 2.552$). Also the mean body-weight of the anoestrous rats of the third group is of the same order of magnitude as the mean body-weights of these latter two groups. It is much higher than either the mean body-weight of the normal-oestrous group or that of the normal-dioestrous group, although the differences are not statistically significant (diff. = 11.8 g., $n = 18$, $t = 2.12$, P is almost 0.05, and diff. = 9.6 g., $n = 18$, $t = 1.83$, P is between 0.1 and 0.05).

These observations show that body-weight increases after spaying, and that the increase is inhibited by large but not by threshold daily doses

of oestrone. The latter finding accords with observations published by Korenchevsky and his collaborators [c.g. Korenchevsky & Dennison, 1934; Korenchevsky, Hall & Ross, 1939; Korenchevsky, Burbank & Hall, 1939] and by Lauson, Heller & Sevringhaus [1937]. The 'stunting' effect of large doses of oestrone is probably mediated through the pituitary [Deanesly, 1939]. It is obvious that depression of hypophyseal activity did not occur in the animals given threshold doses.

Adrenal-weight

The mean adrenal-weight in the normal animals is much smaller than in the corresponding experimental groups. Statistical analysis (Table III) shows that in two cases the difference is not significant by the standards adopted in this study ($P = 0.02$).

Table II. *Mean adrenal-weight (in mg.) under different conditions*

Group 1		Group 2		Group 3	
Normal cycles		Artificial threshold cycles		Animals in constant oestrus or anoestrus	
Oestrus	Dioestrus	Oestrus	Dioestrus	Const. oestrus	Const. anoestrus
48.25	42.25	54.45	47.10	56.70	54.90
± 0.92	± 1.10	± 2.11	± 1.73	± 3.67	± 3.56

Table III. *Comparison of mean adrenal-weights in normal and experimental groups. $P = 0.02$ when $t = 2.552$ (significant differences in Clarendon type).*

Groups compared	Diff. (mg.)	n	t	P
Normal oestrus and artificial oestrus	6.20	18	2.70	0.02-0.01
„ dioestrus and artificial dioestrus	4.85	18	2.37	0.05-0.02
„ oestrus and constant oestrus	8.45	18	2.24	0.05-0.02
„ dioestrus and constant anoestrus	12.65	18	3.40	< 0.01

The comparisons in Table III are between corresponding groups. The quantitative effects of castration appear even more significant statistically when the mean adrenal-weight for the normal dioestrous group is compared with those of the experimental oestrous groups. Thus analysis of the difference (12.2 mg.) between the mean adrenal-weight for the animals in normal dioestrus and that of the animals in the oestrous phase of the artificial threshold oestrous cycle gives the following results: $n = 18$, $t = 5.14$, $P < 0.01$. Here, however, the effect of castration on the adrenals is undoubtedly magnified by that of oestrogenic stimulation (see below).

Table II also shows that the mean adrenal-weight is consistently bigger in the oestrous than in the non-oestrous animals of each group. The differences are analysed in Table IV. With the exception of the third group, in which the variance of adrenal-weight is greatest, the differences are significant. This finding confirms that of our preliminary study made

on a different group of animals, and indicates that the adrenal increases in size not only during normal oestrus, as Andersen & Kennedy [1932] demonstrated, but also in the oestrous phase of the artificial threshold cycle.

Table IV. *Comparison of mean adrenal-weights in oestrous and non-oestrous groups*

Groups compared	Diff. (mg.)	n	t	P
Normal oestrus and normal dioestrus	6.00	18	4.19	<0.01
Artificial oestrus and artificial dioestrus	7.35	18	2.70	0.02-0.01
Constant oestrus and constant anoestrus	1.80	18	0.35	0.8-0.7

Adrenal-weight in relation to body-weight

Andersen & Kennedy [1932] found that in the rat relative adrenal-weight decreases progressively with body-weight, within a body-weight range of 150 to 225 g., while absolute weight increases. Our own observations show that adrenal-weight is positively correlated with body-weight even within the narrow body-weight range (148-196 g.) which our data comprise.

In determining the correlation coefficient, our 60 sets of paired data (body-weight and adrenal-weight) were divided into two groups, the first made up the ten normal oestrous animals and the twenty experimental oestrous animals, and the second of the ten normal and the twenty experimental non-oestrous animals.

The correlation coefficient for adrenal-weight and body-weight in the first group proved to be: $r = 0.500 \pm 0.139$, and in the second group: $r = 0.571 \pm 0.125$. In both cases the values of the coefficient in terms of P (n being 28) was much less than 0.01 (with 25 degrees of freedom $P = 0.01$ when $r = 0.4869$; with 30 degrees of freedom $P = 0.01$ when $r = 0.4487$).

This result implies that a more correct picture of our findings would be provided if our absolute adrenal-weight figures were converted into relative values in order to correct for variations in body-weight.

Adrenal-weight/body-weight ratio

Accordingly, for purposes of comparison, adrenal-weights in each case have been translated into the expression $\frac{x \text{ mg. adrenal} \times 100}{y \text{ g. body-weight}}$, called for simplicity *AW/BW ratio*. The results obtained are given in Table V.

Expressed in this form, the differences in mean adrenal-weights between the oestrous and non-oestrous animals in each group become even more striking (Table VI).

Before discussing the implications of these findings it is necessary to consider certain other data.

Adrenal-volume

As indicated on pp. 285-6, total adrenal-volume and the volume of cortex and medulla were estimated from the areas of projected outlines of every 10th 10μ section. The method is obviously to some extent one of approximation, but the results justify its use. The figures obtained are given in Table VII.

Table V. *Mean relative adrenal-weights (AW/BW ratios) under different conditions*

Group 1		Group 2		Group 3	
Normal cycles		Artificial threshold cycles		Animals in constant oestrus or anoestrus	
Oestrus	Dioestrus	Oestrus	Dioestrus	Const. oestrus	Const. anoestrus
30.33 ± 0.575	26.26 ± 0.582	30.75 ± 0.893	26.49 ± 0.960	34.94 ± 1.960	32.018 ± 1.449

Table VI. *Comparison of mean adrenal/body-weight ratios in different groups*

Groups compared	Diff. (mg.)	n	t	P
Normal oestrus and normal dioestrus	4.066	18	4.98	<0.01
Artificial oestrus and artificial dioestrus	4.256	18	3.25	<0.01
Constant oestrus and anoestrus	2.917	18	1.20	0.3-0.2

Table VII. *Mean adrenal-volume (in mm.³) and volume of cortex and medulla under different conditions*

	Group 1		Group 2		Group 3	
	Normal cycles		Artificial threshold cycles		Animals in constant oestrus or anoestrus	
	Oestrus	Dioestrus	Oestrus	Dioestrus	Const. oestrus	Const. anoestrus
Whole gland	23.73 ± 1.213	19.97 ± 0.900	25.95 ± 1.675	21.91 ± 1.712	26.57 ± 2.349	26.04 ± 2.598
Cortex	22.41 ± 1.151	18.70 ± 0.860	24.68 ± 1.600	20.73 ± 1.650	25.30 ± 2.270	24.68 ± 2.490
Medulla	1.32 ± 0.079	1.27 ± 0.074	1.27 ± 0.082	1.18 ± 0.066	1.27 ± 0.112	1.36 ± 0.120

Although the mean volume of the oestrous adrenal in each group is greater than that of the corresponding non-oestrous adrenal, statistical analysis showed that the difference was significant only in the first group ($P = 0.02$).

Adrenal-volume in relation to body-weight

The possibility that variance in the body-weight figures might be responsible for the lack of statistical significance to the differences in the second and third groups made it necessary to determine whether adrenal-

volume, like adrenal-weight, is positively correlated with body-weight. Analysis showed that it is.

The data were divided into oestrous and non-oestrous groups as in the analysis of the adrenal-weight/body-weight correlation. The correlation coefficient for adrenal-volume and body-weight in the oestrous group proved to be: $r = 0.3896 \pm 0.158$. In this case the value of r in terms of P (n being 28) is approximately 0.02 (it is exactly 0.02 with 25 degrees of freedom when $r = 0.4451$; with 30 degrees of freedom when $r = 0.4093$). The coefficient r for the dioestrous group proved to be 0.4878 ± 0.142 , its value in terms of P being < 0.01 .

Adrenal-volume/body-weight ratio

Since adrenal-volume is positively correlated with body-weight, the volume figures have in each case been translated into the expression $\frac{x \text{ mm.}^3 \times 100}{y \text{ g. body-weight}}$ (called for convenience the AV/BW ratio). The results obtained are given in Table VIII.

Table VIII. *Relative adrenal-volumes (AV/BW ratios) under different conditions*

Group 1 Normal cycles		Group 2 Artificial threshold cycles		Group 3 Animals in constant oestrus or anoestrus	
Oestrus	Dioestrus	Oestrus	Dioestrus	Const. oestrus	Const. anoestrus
15.00	12.40	14.69	12.30	16.27	15.11
± 0.783	± 0.514	± 0.908	± 0.931	± 1.120	± 1.261

Analysis shows that the differences between the oestrous and non-oestrous values in each group are more striking when expressed this way, but that they are still significant only in the normal animals. Since the differences in mean adrenal-weights were significant in both the first and second groups, the results of the analysis of the volume figures suggest either that their variance, due to shortcomings in the method followed in obtaining the volumes, is too great to reveal statistically a real difference, or that adrenal-volume is not correlated with adrenal-weight.

Adrenal-weight in relation to adrenal-volume

Statistical analysis shows that the latter explanation does not hold, since a marked positive correlation exists between adrenal-weight and adrenal-volume both in the 30 oestrous and in the 30 non-oestrous animals. The correlation coefficient in the oestrous group proved to be $r = 0.684 \pm 0.029$, and in the non-oestrous group $r = 0.909 \pm 0.032$. In both cases the values of the coefficient in terms of P (n being 28) is very much less than 0.01.

This analysis implies that the lack of statistical significance in the differences between the artificial oestrous and experimental dioestrous, and constant oestrous and constant anoestrous adrenal-volumes and AV/BW ratios is due to variance in the data introduced by the method followed in obtaining the volume figures.

Changes in the size of cortex and medulla

The data in Table VII show that the volume of the medulla does not fluctuate significantly in or between any of the groups (the small variations are all within the range of observational error, the greatest difference on statistical analysis indicating a P of 0.5). On the other hand the mean volume of the cortex in each oestrous group is greater than that of the corresponding non-oestrous group. The fact that the volume of the medulla does not fluctuate outside the range of observational error suggests that the greater variance of the adrenals of the oestrous as compared with those of the non-oestrous animals is due to variability in the effects which oestrogenic stimulation has upon the cortex.

Although the mean volume of the adrenal cortex in the oestrous rats is in each group greater than that of the corresponding non-oestrous rats, analysis shows that the difference is significant statistically ($P = 0.02$) only in the first group. Conversion of the cortical volume figures to cortical-volume/body-weight ratios increases the degree of statistical significance of the differences, but does not carry them as far as a P value of 0.02.

Cortical and medullary weight-indices

It was shown that in view of the strong positive correlation which exists between body-weight and adrenal-weight, the most convenient way of allowing for fluctuations in body-weight is to express adrenal-weight by the index:

$$\frac{x \text{ mg. adrenal-weight} \times 100}{y \text{ g. body-weight}}.$$

The parts of this index made up by cortex and medulla respectively in any given gland are given by the expressions:

$$\frac{100 (AW)(CV)}{(BW)(AV)} \text{ and } \frac{100 (AW)(MV)}{(BW)(AV)},$$

where AW = total adrenal-weight in mg.

BW = body-weight in g.

AV = total adrenal-volume in mm.³

CV = volume of cortex in mm.³

and MV = volume of medulla in mm.³

This operation presupposes that the specific gravities of the cortex and medulla are approximately equal.

The mean separate weight-indices of the cortex and medulla in each of our three groups of animals are given in Table IX; their differences are analysed in Table X.

Table IX. *Mean cortical and medullary weight-indices under different conditions*

	Group 1		Group 2		Group 3	
	Normal cycles		Artificial threshold cycles		Animals in constant oestrus or anoestrus	
	Oestrus	Dioestrus	Oestrus	Dioestrus	Const. oestrus	Const. anoestrus
Cortex	28.77 ±0.56	24.58 ±0.59	29.25 ±0.88	25.03 ±0.92	33.25 ±1.91	30.32 ±1.42
Medulla	1.69 ±0.07	1.67 ±0.07	1.50 ±0.03	1.46 ±0.06	1.70 ±0.11	1.69 ±0.08

Table X. *Comparison of mean cortical weight-index in different groups*

Groups compared	Diff. (g.)	n	t	P
Normal oestrus and dioestrus	4.19	18	5.191	<0.01
Artificial oestrus and artificial dioestrus	4.22	18	3.323	<0.01
Constant oestrus and anoestrus	2.93	18	1.234	0.3-0.2

It is plain that the mean medullary weight-index of the oestrous rats in each group does not differ significantly from that of the corresponding non-oestrous animals. On the other hand, the cortical weight-index does in the first and second, but not in the third group. This result clearly indicates that the increase in size of the adrenal both in the normal and artificial threshold oestrous cycle is due to cortical growth. This point is also brought out by converting the figures for the volumes of the cortex and medulla in each gland to cortex/medulla ratios (Tables XI and XII).

Table XI. *Mean cortex/medulla ratio under different conditions*

Group 1		Group 2		Group 3	
Normal cycles		Artificial threshold cycles		Animals in constant oestrus or anoestrus	
Oestrus	Dioestrus	Oestrus	Dioestrus	Const. oestrus	Const. anoestrus
17.30 ±0.74	15.00 ±0.82	19.33 ±0.59	17.00 ±0.65	20.19 ±1.46	18.54 ±0.77

Table XII. *Comparison of mean cortex/medulla ratios in different groups*

Groups compared	D.F. (c)	n	t	P
Normal oestrus and dioestrus	2.39	18	2.486	0.02
Artificial oestrus and dioestrus	2.53	18	2.442	0.02-0.01
Constant oestrus and anoestrus	1.65	18	1.004	0.2

Fluctuation in adrenal-size during the oestrous cycle

Our observations fully confirm Andersen & Kennedy's [1932] finding that the adrenals of the rat increase in size during normal oestrus, and that the change is due to growth of the cortex. They also show that identical changes occur during threshold artificial oestrous cycles. Since it is hardly likely that the adrenals would react cyclically to a constant daily threshold dose of oestrone, it is reasonable to suppose that the cycle of changes which they undergo under these conditions in spayed animals represent an 'inherent' change which proceeds independently of the ovaries and of oestrogenic stimulation. The latter undoubtedly has an effect on the adrenals (see below), but it is not an effect which expresses itself in this cyclical way.

It is known that the cells of the cortex are continually degenerating in the region of the medulla, and that fresh cells are always migrating from the outer zones (according to Zwemer, Wotton & Norkus [1938] from the indifferent cells of the capsule) towards the centre [see Grollman, 1936]. It is possible that the increase in size of the cortex at oestrus represents either a peak of cellular formation or an increase in cell size. This point is considered in detail in a later section, as is also the problem of the continuation of the change after hypophysectomy.

Effects of oestrogenic stimulation and spaying on the adrenal cortex

Most workers who have investigated the problem report that oestrogenic stimulation leads to an increase in the size of the rat adrenal [e.g. Andersen, 1934; Korenchevsky & Dennison, 1934, 1935; Selye, Collip & Thomson, 1935; Ellison & Birch, 1936; Deanesly, 1939] and evidence provided by Selye *et al.* [1935] and Ellison & Birch [1936] indicates that the influence of oestrogenic stimulation on the adrenals is mediated through the anterior pituitary.

Spaying under certain conditions also leads to adrenal enlargement. The problem was reviewed in detail by Andersen & Kennedy [1933], and their own observations showed that spaying before maturity (at 20-21 days) had no effect on mean adrenal-weight up to the 85th day. On the other hand, the adrenals of rats spayed after sexual maturity had been reached (between 53 and 73 days) had hypertrophied a week after gonadectomy; at 3, 6 and 8 weeks after the operation, however, they weighed about 30% less than the mean adrenal-weight for normal oestrous females. Contrary results are reported by Waterman [1939], who found a significant decrease in the weight of the adrenals of mature female rats one week after spaying (49 ± 2.1 mg., 14 spayed animals, as compared with 66 ± 2.6 mg., 23 normal animals). Observations published by Blumenfeld [1939] refer to adrenals removed from rats 8 weeks and 20 weeks after ovariectomy (performed

during the 4th week of life) and to adrenals removed 12 weeks after spaying (performed at 3 months). In all three groups the adrenals were significantly lighter than normal; 8 weeks after spaying the mean difference was 8.3% and after 20 weeks 33.4%. The diminution in size was due to cortical atrophy, the zona fasciculata being affected first, and all three zones later. Lauson *et al.* [1937] also observed that the adrenals of female rats are slightly atrophied 20 days after spaying. In male rats, however, castration leads to an increase in the size of the adrenals [e.g. Korenevsky, 1930; Hall & Korenevsky, 1938]. In the only three other species that have been studied extensively (the rabbit, the guinea-pig and the mouse) gonadectomy leads to an increase in adrenal-weight [see Blumenfeld, 1939; Deanesly, 1938].

There seems to be fairly general agreement that in the rat spaying leads, after three weeks, to progressive involution of the adrenals; few workers, however, have paid attention to the precautions, indicated by Andersen & Kennedy [1933], to use as controls in such studies animals in known phases of the reproductive cycle. Although the mean adrenal-weight of a group of animals after gonadectomy may, for example, be less than that of a control group collected in oestrus, it could nevertheless be greater than that of a control series removed in dioestrus.

So far as the immediate effects of gonadectomy on the adrenals are concerned, our observations support those of Andersen & Kennedy and disagree with those of Waterman. Thus the mean adrenal-weight in the 10 rats which received no treatment for 14 days after spaying (Group 3) is significantly greater than that of the 10 normal animals in dioestrus (Group 1, Tables II and III). As already noted, this relationship holds, although not in so striking a way, between the mean adrenal-weight of both the oestrous and dioestrous normal animals and the mean weights of all the other corresponding experimental groups. In the case of this comparison, however, evaluation of the effects of castration is made difficult because of the oestrone treatment the animals received, a procedure which introduces a second variable into the problem. That the increase in the size of the adrenal immediately after spaying is due to hypertrophy of the cortex is indicated, first, by the fact that the absolute values for the volume of the medulla in the normal and experimental groups do not vary outside the range of observational error, and second, by the fact that the mean cortex/medulla ratio of the 10 normal dioestrous rats is significantly greater than that of the 10 anoestrous animals of Group 3 (diff. = 3.54, $n = 18$, $t = 3.159$, $P < 0.01$).

It was noted earlier on (pp. 288-9) that while the mean adrenal-weights of both the normal oestrous and artificial oestrous groups were significantly greater than those of their corresponding dioestrous groups, that of the 10

rats which were kept in constant oestrus was greater, but not significantly so, than that of the 10 anoestrous rats in the same group. It is conceivable that the smaller relative difference in this group, as compared with that in the second group, was due to some antagonism between the effects of castration and those of the relatively large doses of oestrone the animals received. Thus Korenchevsky, Hall & Ross [1939] found that the absolute size of the hypertrophied adrenals of castrated male rats decreased as a result of prolonged oestrogenic treatment although their relative size per unit body-weight increased somewhat. Moreover, in a later study Korenchevsky, Burbank & Hall [1939] observed that while the adrenal-weights of spayed female rats increased as a result of low doses of oestradiol they decreased after treatment with high doses (99 and 200 μ g. weekly). In an earlier study Korenchevsky & Dennison [1935] also found that 18 μ g. of oestrone given daily for 43 days had no effect on the already hypertrophied adrenals of male rats. Why this should be so is not at present obvious, but it is an observation which accords with the trend of our own findings, and which offers some explanation for the fact that the relative difference between the mean adrenal-weights in the experimental oestrous cycle is greater than that between those of the constant oestrus and uninjected animals of Group 3.

Changes in the cortical zones

An attempt was made, by the method outlined on pp. 285-6, to determine the volumes of the different cortical zones in the 60 animals used in this study. This particular method was improvised and adopted, in spite of the shortcomings which have already been indicated, because the literature

Table XIII. *Mean volumes (in mm.³) of cortical zones under different conditions*

Zone	Group 1		Group 2		Group 3	
	Normal Cycles		Artificial threshold cycles		Animals in constant oestrus or anoestrus	
	Oestrus	Dioestrus	Oestrus	Dioestrus	Const. oestrus	Const. anoestrus
Glomerulosa	1.66 ± 0.13	1.50 ± 0.12	1.94 ± 0.10	1.60 ± 0.19	1.76 ± 0.35	1.59 ± 0.09
Fasciculata	9.04 ± 0.37	7.36 ± 0.43	11.93 ± 0.60	9.47 ± 0.58	10.22 ± 0.78	9.80 ± 0.57
Reticularis	3.51 ± 0.34	2.69 ± 0.13	3.90 ± 0.34	3.22 ± 0.47	3.88 ± 0.34	3.98 ± 0.48

provided no guide to a more immediately practicable procedure for obtaining quantitative information on the problem. The results of the application of the method are given in Table XIII.

In every case except one (zona reticularis of Group 3), the mean volumes for the different zones in the oestrous animals are larger than the means in the corresponding non-oestrous animals. Table XIV shows that the difference in the exceptional case is not significant statistically, and that in spite of the obvious shortcomings of the method used in determining the zone volumes, the differences between the oestrous and non-oestrous values for the fasciculata are significant in Groups 1 and 2. This fact suggests that the increased size in the adrenal cortex at oestrus both in the normal and artificial threshold oestrous cycle, is due in greater part to changes in the zona fasciculata. The data also suggest that the degree of change in the zona glomerulosa is less than that in the zona reticularis.

Table XIV. *Comparison of mean zone-volumes in different groups*

Zone	Phases compared	Diff.	n	t	P
Glomerulosa	Normal oestrus and dioestrus	0.159	18	0.904	0.4-0.3
	Artificial oestrus and dioestrus	0.342	18	1.617	0.1-0.2
	Constant oestrus and anoestrus	0.162	18	0.448	0.7-0.6
Fasciculata	Normal oestrus and dioestrus	1.681	18	2.953	<0.01
	Artificial oestrus and dioestrus	2.464	18	2.974	<0.01
	Constant oestrus and anoestrus	0.424	18	0.441	0.7-0.6
Reticularis	Normal oestrus and dioestrus	0.815	18	2.235	0.05-0.02
	Artificial oestrus and dioestrus	0.683	18	1.873	0.1-0.05
	Constant oestrus and anoestrus	-0.105	18	0.179	0.9-0.8

Changes in cell-size

In order to determine whether the increase in the size of the adrenal cortex during normal and artificial oestrus (and immediately after spaying), is associated with an increase in the size of the cortical cells, estimates were made of the mean cell-volume in each zone and also in the medulla of every adrenal gland. The cortical cells are usually described as being spherical or polyhedral. In determining their size they have been regarded as spherical, as (i) the cells are probably in any event squashed spheres, and (ii) it would have been practically impossible to estimate the volume of a polyhedral cell.

The mean diameter of 30 cells chosen at random in each zone (including the medulla) of each of the adrenal glands was measured with a micrometer eye-piece. Cell volume was calculated from the formula $\frac{4}{3}\pi r^3$. Reticularis cells were measured in the inner band of the zona reticularis to avoid confusion with the fasciculata cells.

The values obtained are given in Table XV.

It will be seen by inspection that such variations as occur in the size of the cells of the zona glomerulosa in the oestrous and non-oestrous phases of each group are within the range of observational and sampling error. It

follows therefore that cell size in this zone remains fairly constant during oestrous and non-oestrous phases. On the other hand, mean cell-size in the zona fasciculata during oestrous is significantly greater than in non-oestrous phases (Table XVI).

Table XV. *Mean cell-size (in μ^3) in the different adrenal zones under different conditions*

Zone	Group 1		Group 2		Group 3	
	Normal cycles		Artificial threshold cycles		Animals in constant oestrus or anoestrus	
	Oestrus	Dioestrus	Oestrus	Dioestrus	Const. oestrus	Const. anoestrus
Glomerulosa	360.8 ± 12.33	373.0 ± 11.86	498.7 ± 19.97	481.0 ± 21.77	373.9 ± 25.10	375.5 ± 12.62
Fasciculata	2435.0 ± 37.25	1608.0 ± 53.42	2576.0 ± 88.68	1974.0 ± 102.1	2282.0 ± 94.92	1840.0 ± 64.41
Reticularis	330.8 ± 15.63	297.4 ± 14.07	429.6 ± 20.01	448.8 ± 27.78	362.9 ± 21.37	375.1 ± 9.95
Medulla	1382.0 ± 69.55	1387.0 ± 42.06	1621.0 ± 48.36	1568.0 ± 70.11	1520.0 ± 34.64	1375.0 ± 41.13

Table XVI. *Comparison of mean cell-size in different zones under different conditions (the larger value in each case is in italics)*

Zone	Groups compared	Diff. (μ^3)	n	t	P
Fasciculata	<i>Normal oestrus</i> and dioestrus	827.0	18	12.73	<0.01
	<i>Artificial oestrus</i> and dioestrus	602.0	18	4.461	<0.01
	<i>Constant oestrus</i> and anoestrus	442.0	18	3.860	<0.01
Reticularis	<i>Normal oestrus</i> and dioestrus	33.4	18	1.591	0.2-0.1
	<i>Artificial oestrus</i> and dioestrus	59.2	18	1.732	0.1
	<i>Constant oestrus</i> and anoestrus	12.2	18	0.518	0.6
Medulla	<i>Normal oestrus</i> and dioestrus	5.0	18	0.062	1.0-0.9
	<i>Artificial oestrus</i> and dioestrus	53.0	18	0.623	0.6-0.5
	<i>Constant oestrus</i> and anoestrus	145.0	18	2.700	0.02-0.01

The differences in mean size between the oestrous and non-oestrous reticularis cells follow no definite rule, and none is significant statistically. On the other hand, experimental oestrogenic stimulation appears to lead to an increase in the size of the medullary cells, the increase being significant statistically in the group injected for 14 days with 50 μ g. of oestrone daily. What the significance of this change is remains to be determined; to our knowledge there has been no published indication hitherto that the adrenal medulla is affected by oestrogens.

The results of the analysis thus show that the increase in size of the adrenals during oestrus in normal and artificial threshold oestrous cycles is due to an increase in the size of the cortex, and in particular of the zona fasciculata, and that this increase is associated with a marked increase in

the size of the fasciculata cells. It does not appear to be associated, as the following section shows, with an increase in the number of cortical cells.

The number of cortical cells

An approximation of the number of cells in the adrenal cortex was obtained by the following formula, derived in the manner shown.

If v = measured volume of cortex (pp. 285-6),

x = estimated volume of zona glomerulosa,

y = " " " fasciculata,

z = " " " reticularis,

x' = " " glomerulosa cell,

y' = " " fasciculata cell,

and z' = " " reticularis cell.

Then (i) $\frac{vx}{(x+y+z)(x')} =$ number of glomerulosa cells,

(ii) $\frac{vy}{(x+y+z)(y')} =$ " fasciculata cells,

(iii) $\frac{vz}{(x+y+z)(z')} =$ " reticularis cells,

and (iv) total number of cortical cells is

$$\frac{v}{x+y+z} \left(\frac{x}{x'} + \frac{y}{y'} + \frac{z}{z'} \right),$$

$\frac{v}{x+y+z}$ being a correction factor for the method of obtaining the partial volumes of the cortex.

Since the size of the adrenal cortex is correlated with body-weight (pp. 290-1), a corrected index of cell number is given by the expression

$$\frac{v}{BW(x+y+z)} \left(\frac{x}{x'} + \frac{y}{y'} + \frac{z}{z'} \right)$$

where BW is the body-weight in g.

The data yielded by the application of this formula are given in Table XVII.

Table XVII. *Mean number ($\times 10^6$) of cortical cells per g. body-weight under different conditions*

Analysis of these figures (Table XVIII) shows that there is no significant variation in the number of cortical cells in the oestrous and non-oestrous animals of the different groups. Consequently, it follows that the increase in size of the adrenals during oestrus in normal and artificial threshold oestrous cycles is essentially due to hypertrophy of the cells of the zona fasciculata.

Table XVIII. *Comparison of mean numbers ($\times 10^6$) of cortical cells in different groups (larger values in each comparison in italics)*

Phases compared	Diff.	n	t	P
<i>Normal oestrus</i> and normal dioestrus	0.0095	18	0.455	0.7-0.6
<i>Artificial oestrus</i> and art. dioestrus	0.0201	18	1.420	0.2-0.1
Constant oestrus and <i>const. anoestrus</i>	0.0035	18	0.150	0.9-0.8
<i>Normal oestrus</i> and artificial oestrus	0.0509	18	2.438	0.05-0.02
<i>Normal oestrus</i> and constant oestrus	0.0040	18	0.178	0.9-0.8
Artificial oestrus and <i>const. oestrus</i>	0.0469	18	2.874	0.01
<i>Normal dioestrus</i> and art. dioestrus	0.0615	18	4.351	<0.01
Normal dioestrus and <i>const. anoestrus</i>	0.0090	18	0.242	0.9-0.8
Artificial dioestrus and <i>const. anoestrus</i>	0.0705	18	3.260	<0.01

The number of medullary cells

The number of medullary cells, which were estimated by dividing the estimated mean volume of medullary cell in each gland into the observed total medullary volume (pp. 290-1), also fails to vary significantly in the oestrous and non-oestrous animals of the different groups (Tables XIX and XX). The values are given as the number of cells $\times 10^3$ per g./body-weight.

Table XIX. *Mean number ($\times 10^3$) of medullary cells per g./body-weight under different conditions*

Normal oestrus	= 6.158 \pm 0.5081
„ dioestrus	= 5.703 \pm 0.2888
Artificial oestrus	= 4.484 \pm 0.3494
„ dioestrus	= 4.351 \pm 0.3377
Constant oestrus	= 5.173 \pm 0.3442
„ anoestrus	= 5.789 \pm 0.4301

Table XX. *Comparison of mean numbers ($\times 10^3$) of medullary cells in different groups (the larger value in each case is in italics)*

Phases compared	Diff.	n	t	P
<i>Normal oestrus</i> and normal dioestrus	0.455	18	0.780	0.5-0.4
<i>Artificial oestrus</i> and art. dioestrus	0.133	18	0.274	0.8-0.7
Constant oestrus and <i>const. anoestrus</i>	0.616	18	1.120	0.3-0.2
<i>Normal oestrus</i> and artificial oestrus	1.674	18	2.721	0.02-0.01
<i>Normal oestrus</i> and constant oestrus	0.985	18	1.608	0.2-0.1
Artificial oestrus and <i>const. oestrus</i>	0.689	18	1.409	0.2-0.1
<i>Normal dioestrus</i> and art. dioestrus	1.352	18	3.047	<0.01
Normal dioestrus and <i>const. anoestrus</i>	0.086	18	0.166	0.9-0.8
Art. dioestrus and <i>const. anoestrus</i>	1.438	18	2.632	0.02-0.01

*Effects of oestrogenic stimulation and spaying on zone volumes,
cell-size and cell-number*

Our data indicate, as already shown (p. 294), that at the end of the second week after spaying the adrenal is increased in size. Since the volume of medulla remains constant (Table IX) the increase must be attributed to changes in the cortex. Table XIII shows that all three cortical zones in the two spayed groups of animals (Groups 2 and 3) are consistently larger than the corresponding zones in the normal group (Group 1). The comparison is complicated, except in that of the normal dioestrous animals and the untreated animals of Group 3, by the fact that the experimental groups were given oestrone daily.

Comparison shows, however, that the zona fasciculata and zona reticularis in the spayed untreated rats of Group 3 are significantly larger than in the normal dioestrous animals of Group 1 (Table XXI). The difference in the size of the zona glomerulosa is not significant. The finding indicates that adrenal enlargement in the first two weeks after spaying is due to changes in the zona fasciculata and zona reticularis.

Table XXI. *Comparison of zone volumes in normal dioestrous (Group 1) and spayed anoestrous rats (Group 3)*

Zones compared	Diff. (mm. ³)	n	t	P
Glomerulosa	0.094	18	0.654	0.6-0.5
Fasciculata	2.441	18	3.441	<0.01
Reticularis	1.292	18	2.610	0.02-0.01

Statistical analysis of the differences in cell-size in the different groups (Table XV) yields interesting results (Table XXII).

The mean sizes of the glomerulosa and medullary cells in animals which received no treatment for 14 days after spaying are the same as in the normal dioestrous group. On the other hand, those of the fasciculata and reticularis cells are significantly larger, a finding which indicates that adrenal enlargement after spaying is due to hypertrophy of these cells.

Furthermore, the glomerulosa cells of both sets of animals in the second experimental group are significantly larger than those of the first and third groups, a finding which suggests that these cells increase in size as a result of stimulation by threshold doses of oestrone but not as a result either of castration or of relatively large doses of oestrone.

In the case of the fasciculata cells the results are somewhat similar. In the oestrous animals large doses of oestrone appear to lead to a smaller increase than threshold doses (the differences, however, are not significant statistically). In the non-oestrous animals the stimulating effect of threshold doses seems even more apparent, as shown by the significant differences

in fasciculata cell-size between the normal animals of Group 1 and the injected animals of Group 2; in this case the comparison is complicated by the fact that spaying alone results in cellular hypertrophy.

The smaller stimulating effect of the large doses of oestrone on cell-size is equally well shown in the reticularis cells, for here again the cells are smaller (though not significantly so) in the animals given large doses (Group 3) than in the animals given threshold doses (Group 2). The reticularis cells of the latter group are also significantly larger than those of

Table XXII. *Comparison of mean cell-size in different groups*
(the larger value in each case in *italics*)

Zono	Groups compared	Diff. (μ^3)	n	t	P
Glomerulosa	Norm. oestrus and <i>art. oest.</i>	137.9	18	5.893	<0.01
	<i>Art. oest.</i> and const. oest.	124.8	18	3.897	<0.01
	Normal dioest. and <i>art. dioest.</i>	108.0	18	4.363	<0.01
	<i>Art. dioest.</i> and const. anoest.	105.5	18	4.200	<0.01
Fasciculata	Norm. oest. and <i>art. oest.</i>	141.0	18	1.469	0.2-0.1
	<i>Norm. oest.</i> and const. oest.	153.0	18	1.503	0.2-0.1
	<i>Art. oest.</i> and const. oest.	294.0	18	2.268	0.05-0.02
	Norm. dioest. and <i>art. dioest.</i>	366.0	18	3.182	<0.01
	Norm. dioest. and <i>const. anoest.</i>	232.0	18	2.778	0.02-0.01
Reticularis	<i>Art. dioest.</i> and const. anoest.	134.0	18	1.112	0.3-0.2
	Normal oest. and <i>art. oest.</i>	98.8	18	3.898	<0.01
	Norm. oest. and <i>const. oest.</i>	32.1	18	1.214	0.3-0.2
	<i>Art. oest.</i> and const. oest.	66.7	18	2.282	0.05-0.02
	Norm. dioest. and <i>art. dioest.</i>	191.4	18	6.158	<0.01
Medulla	Norm. dioest. and <i>const. anoest.</i>	77.7	18	4.522	<0.01
	<i>Art. dioest.</i> and const. anoest.	113.7	18	3.861	<0.01
	Norm. oest. and <i>art. dioest.</i>	239.0	18	2.827	0.01
	Norm. oest. and <i>const. oest.</i>	138.0	18	1.779	0.1-0.05
	<i>Art. oest.</i> and const. oest.	101.0	18	1.701	0.1
	Norm. dioest. and <i>art. dioest.</i>	181.0	18	2.217	0.05-0.02
	<i>Norm. dioest.</i> and const. anoest.	12.0	18	0.204	0.9-0.8
	<i>Art. dioest.</i> and const. anoest.	193.0	18	2.378	0.05-0.02

normal animals (Group 1) and of the animals of the third group which were given no treatment, a finding which indicates clearly that threshold doses of oestrone lead to hypertrophy of the reticularis cells.

Analysis of the changes in the size of the medullary cells points to the same general conclusions.

It thus appears that gonadectomy leads to an increase in the size of the fasciculata and reticularis cells, and that threshold doses of oestrone result in enlargement not only of these cells but also of the cells of the glomerulosa and medulla. On the other hand, large doses of oestrone have a less stimulating effect (fasciculata and medulla), and sometimes a depressing effect (glomerulosa and reticularis), on cell-size in all the zones of the adrenal.

As already observed, the greater size of the adrenal cortex during oestrus

both in the normal and in the artificial threshold cycle is not associated with an increase in the estimated number of cortical cells (Tables XVII and XVIII). Analysis also shows that the number does not increase significantly at the end of 14 days after spaying (comparison of normal dioestrous and constant anoestrous groups in Tables XVII and XVIII). On the other hand it appears to decrease significantly as a result of stimulation by threshold doses of oestrone, although not as a result of stimulation by large doses (Tables XVII and XVIII). The estimated number of medullary cells fluctuates in the same way as that of the cortical cells (Tables XIX and XX).

The following picture of the quantitative changes which take place in the adrenal is obtained when the results of the whole analysis are combined. The adrenal increases in size significantly both during normal oestrus and in the oestrous phase of artificial threshold cycles. The change is due to cortical hypertrophy and in particular to growth of the zona fasciculata. Less-marked growth occurs in the zona reticularis, and still less in the zona glomerulosa. These changes are not associated with significant alterations in the estimated total number of cortical cells. On the other hand, the fasciculata cells increase in size significantly and this therefore appears to be the essential change that takes place in the adrenal at oestrus.

A fortnight after spaying the adrenals are also enlarged, the change occurring in the cortex and being due in particular to growth in the zona fasciculata and zona reticularis. The changes in these zones are due to cellular swelling and not to an increase in the total number of their constituent cells.

Oestrogenic stimulation also has specific effects on the adrenals. Threshold doses of oestrone lead to a significant and marked increase in the size of the cortex while large doses have a much slighter stimulating effect. The stimulating effect of threshold doses is associated with swelling of the glomerulosa, fasciculata and reticularis and medullary cells and apparently with a decrease in the total number of cortical cells. Larger doses of oestrone (50 μ g. over a period of 14 days), retain their stimulating effect on the size of fasciculata and medullary cells but lose it on the glomerulosa and reticularis cells. They have no effect on the total number of cortical cells.

Histological changes

This picture of the quantitative changes which occur in the adrenal cortex during the normal oestrous and during the artificial threshold cycle, after spaying and after oestrogenic stimulation, can be briefly correlated with direct histological observation.

During the normal cycle, as originally observed by Andersen & Kennedy [1932], no changes occur in the medulla. During oestrus the zona glomerulosa is narrower and less clearly demarcated from the fasciculata than in dioestrus. The cells of the zona fasciculata are enlarged and vacuolated during oestrus, as are the nests of eosinophilic cells between the reticularis and medulla. The latter appear to be more numerous during dioestrus.

In many specimens the reticularis appears to be wider and to contain more numerous and dilated (blood) spaces and more degenerated cells in oestrus than in dioestrus. The adrenal capsules also appear to be thicker at oestrus.

Microscopic examination suggests that the changes which take place during the artificial threshold cycle are of the same kind, with the one exception that the zona fasciculata appears more compact during artificial than in normal oestrus.

Most published observations, as noted, indicate that the adrenal of the female rat decreases in size after spaying, although no observations, except some of Andersen & Kennedy's, refer to adrenals removed less than 3 weeks after spaying. The decrease that occurs after 3 weeks is particularly associated, according to Blumenfeld [1939], with a decrease in the size of the zona fasciculata, the cells of which are smaller than normal, and show more signs of degeneration. The rate of regeneration, however, is as great, or greater, than normal. Moreover, according to Blumenfeld, the total number of fasciculata cells is not decreased after spaying, a conclusion which he reached by estimating the number of nuclei in a given zone.

Our own observations agree with those of Blumenfeld that there is no alteration in cell-number after spaying, but differ in so far as signs of cellular degeneration are not prominent 2 weeks after spaying. At that time no definite changes occur in the juvenile-zone cells. None was observed by Howard [1938] in the castrated male rat.

The only histological observations regarding the effects of oestrogenic stimulation to which reference need be made is the greater hyperaemia of the cortex in injected as compared with control animals. Large haemorrhagic areas were present in the adrenals of 3 of the 10 spayed females of Group 3 which were given 50 μ g. of oestrone daily for 14 days after spaying.

Changes in the adrenals of hypophysectomized spayed rats

Hypophysectomy, as had been fully established, leads to involution of the adrenals. The involution is entirely due to changes which take place in the cortex, since the volume of the medulla remains relatively unchanged. According to Cutuly [1936], the adrenal cortex of the female rat loses approximately 73% of its weight in 30 days after hypophysectomy. Perla [1935] notes that the most striking immediate change which occurs in the

adrenal cortex after hypophysectomy is haemorrhage into the reticularis, the cells of which become vacuolated and swollen. Occasionally, where haemorrhage is less intense, the inner cortical cells appear shrunken. Haemorrhagic changes, according to Perla, precede cellular atrophy.

Our own findings, as shown in Table XXIII, agree with the observation that the decrease in the size of the adrenals after hypophysectomy is due to cortical involution, and the extent of involution that occurred in our animals was closely similar to that noted by Cutuly. The adrenals, however, were removed too long after hypophysectomy to demonstrate the immediate haemorrhagic changes described by Perla.

Differentiation of the adrenal cortex into its normal zones, as originally observed by Smith [1927, 1930], is difficult after hypophysectomy. The fully involuted adrenal cortex is a relatively narrow cap to a healthy medulla. It is divided into three zones, of which the middle is the narrowest. The outer zone consists of short columns of cells with round nuclei, scanty cytoplasm and indefinite boundaries. The middle narrow zone consists of very tightly packed cells with deep blue-staining irregular nuclei and with indistinguishable cytoplasm and cell boundaries. The inner wide zone stains very lightly, and consists mainly of degenerated cells separated by relatively wide spaces, dilated sinusoids, and collections of old blood pigment. No juvenile cells are to be seen in the peripheral parts of the medulla. The capsule of the gland is relatively thick.

In comparison with a normal gland the outer zone would appear to be the glomerulosa, and the middle zone the deeply staining cells which usually intervene between the glomerulosa and the fasciculata. In size the outer cortical cells are similar to normal glomerulosa cells. The inner degenerated cells would seem to represent the zonae fasciculata and reticularis together.

The adrenal glands were removed from 5 spayed and hypophysectomized rats during the oestrous phase of an artificial threshold cycle, and from 5 during the dioestrous phase. The mean interval between hypophysectomy and removal of the adrenals was the same in both groups. The specimens were serially sectioned and measured in the same way as the other glands described above. The number of data are too few for statistical analysis, but Table XXIII gives the mean values obtained in comparison with the mean values for spayed, but otherwise normal animals, in the oestrous and dioestrous phases of threshold artificial cycles.

Comparison of the figures in the first two columns of Table XXIII shows that the adrenal gland fluctuates in size during artificial threshold cycles in hypophysectomized-spayed animals in the same way as in nonhypophysectomized-spayed animals. The data also demonstrate the considerable degree of involution that occurs in the adrenals after hypophysectomy.

Unfortunately, owing to their very poor definition it was impossible to measure satisfactorily the size of the cortical cells in the hypophysectomized animals, and it was also impossible, because of their profound alterations, to follow changes in the various zones. The dimensions of the outer cortical cells in the adrenals from the hypophysectomized animals were, as noted above, similar to those of normal glomerulosa cells.

Table XXIII. *Mean values for dimensions of adrenals in oestrous and dioestrous spayed and spayed-hypophysectomized rats*

	Hypophysectomized-spayed rats		Spayed rats	
	Oestrus	Dioestrus	Oestrus	Dioestrus
Body-weight	135.0 g.	124.4 g.	177.0 g.	178.4 g.
Adrenal-weight	17.2 mg.	13.0 mg.	54.45 mg.	47.10 mg.
<i>A</i> <i>W</i> / <i>B</i> <i>W</i> ratio	12.65	10.44	30.75	26.49
Adrenal volume	7.93 mm. ³	7.75 mm. ³	25.95 mm. ³	21.91 mm. ³
Cortical volume	6.65 mm. ³	6.54 mm. ³	24.68 mm. ³	20.73 mm. ³
Medulla volume	1.28 mm. ³	1.21 mm. ³	1.27 mm. ³	1.18 mm. ³

It is of interest that threshold oestrogenic stimulation did not lead to an increase in the size of the adrenals of the hypophysectomized animals. As noted above, it did in the non-hypophysectomized animals. This observation lends weight to the view that the effects of oestrogenic stimulation on the adrenals are mediated through the anterior lobe of the pituitary. In so far as the effects of oestrogenic stimulation on the adrenal cortex are dependent on the presence of the pituitary, this observation also corroborates the view that the cyclical changes which occur in the adrenals during the threshold artificial cycle are not due to injected oestrogen.

DISCUSSION

The data which have been analysed above show that the adrenal cortex of spayed rats which are injected with daily threshold doses of oestrone, undergoes cyclical fluctuations in size, in periods of approximately 5 days. Considerations that have been advanced indicate that these changes are not due to the cumulative action of the injected oestrone. Since they take place in the absence of the ovaries, it is unlikely that the approximately five-day cycle which occurs in the size of the adrenal cortex during the normal oestrous cycle is merely a reflection of the cyclical changes that take place in the gonads.

It has also been shown that the adrenal changes continue after removal of the pituitary, through which it has been demonstrated oestrogens influence the adrenals. The evidence available thus far suggests, therefore, that, due to what for the moment must be described as an 'inherent rhythm', cyclical changes take place in the adrenals in period with the phases of the normal oestrous cycle.

These changes are correlated with the periodic appearance of oestrus in spayed rats that are injected with threshold doses of oestrogen, in the same way that the adrenal changes which take place in the normal animal are correlated with the normal cycle of changes in the accessory reproductive organs. As already indicated, it would seem that the adrenals have a direct influence on the accessory reproductive organs.

Three possible mechanisms have been suggested to explain the nature of this influence. The first is that cyclical activity of the adrenals leads to the cyclical hydration and dehydration of the reproductive tract and that such changes determine variations in the responsiveness of the organs to oestrogenic stimulation. At present there is no direct evidence which supports such a view; the problem is, however, amenable to experimentation.

The second possibility is that the adrenal cortex produces a sub-threshold amount of oestrogenic hormone, the amount produced varying cyclically and becoming greater at times of oestrus. The effective oestrogen acting on the reproductive tract of a spayed rat injected daily with threshold doses of oestrogen would thus be the introduced oestrogen supplemented daily by the oestrogen produced by the adrenals. Either source alone would be insufficient to produce oestrous changes in the accessory organs, which would occur when the adrenal cycle of oestrogen production was at its peak. The fact that the adrenalectomized-spayed animals in our series generally required more oestrone to produce an oestrous reaction in the vagina supports this view. Another fact which lends it support is the observation that oestrogenic hormone can be extracted from the adrenal cortex [Engelhart, 1930; Callow & Parkes, 1936; Beall, 1939], and that normally the adrenals are probably the source of the oestrogenic hormone which is found in the urine of gonadectomized men and women [Parkes, 1937].

The third possibility is that the adrenal cortex cyclically produces androgenic and/or progestational hormone, and that this endogenous hormone neutralizes the effect of the introduced hormone during the dioestrous phase of the artificial threshold cycle. Alternatively, such hormone may be produced continuously but in amounts which vary cyclically. In favour of this view is the fact that both androgenic and progestational hormone can be extracted from the adrenal cortex [Callow & Parkes, 1936; Callow, 1938; Beall & Reichstein, 1938] and that the adrenal cortex is responsible for the androgenic hormone which is found in the urine of gonadectomized men and women [Parkes, 1937]. Further investigation is required to decide which, if any, of these possible mechanisms is the determining one.

SUMMARY

1. The adrenal glands of 10 normal oestrous and 10 normal dioestrous rats, of 10 spayed rats in the oestrous phase, and of 10 in the dioestrous

phase of an artificial threshold oestrous cycle, and of 10 spayed rats after 14 daily injections of 50 μ g. of oestrone started the day after the operation, and of 10 untreated spayed rats 15 days after gonadectomy, were studied by quantitative methods.

2. Body-weight did not differ significantly in the oestrous and non-oestrous animals of any experimental group. Analysis showed, however, that body-weight increases after spaying and that the increase is inhibited by large, but not by threshold doses of oestrone.

3. Mean adrenal-weight is consistently greater in the oestrous than in the non-oestrous animals of each group, the differences being significant in all but the third. This finding indicates that the adrenal increases in size not only during normal oestrus, but also in the oestrous phase of the artificial threshold cycle.

4. Adrenal-weight is positively correlated with body-weight, and the differences between the adrenals in the oestrous and non-oestrous animals of each group appear greater when they are expressed as adrenal/body-weight ratios.

5. Similar differences occur between the mean values obtained for adrenal volume in the different groups, which is correlated with body-weight and with adrenal-weight.

6. Analysis of the change shows that the adrenal medulla remains more or less constant in the oestrous and non-oestrous phases of each group, and that the increase in the size of the oestrous adrenal is due to growth in the cortex.

7. The mean adrenal size of the 10 animals which received no treatment in the fortnight after spaying was significantly greater than normal. The change is due to hypertrophy of the cortex.

8. Estimates of the volumes of the different cortical zones indicated that the increased size of the adrenal cortex at oestrus, both in the normal and artificial cycle, is due in greater part to changes in the zona fasciculata. The degree of change in the zona glomerulosa is less than in the zona reticularis.

9. Cell-size in the zona glomerulosa remains fairly constant during oestrous and non-oestrous phases. On the other hand, mean cell-size in the zona fasciculata is significantly greater in oestrous than in non-oestrous phases.

10. Large doses of oestrone appear to stimulate an increase in the size of the medullary cells.

11. No significant variation occurs in the estimated number of cortical cells in the oestrous and non-oestrous animals of the different groups.

12. The increased size of the adrenals of untreated animals after spaying is due particularly to growth in the zona fasciculata and zona reticularis.

The changes in these zones are due to cellular swelling and not to an increase in the total number of their constituent cells.

13. Threshold doses of oestrone lead to a marked increase in the size of the cortex, while large doses have a much lesser stimulating effect.

14. The adrenal glands of hypophysectomized-spayed rats fluctuate in size during artificial threshold cycles in the same way as in non-hypophysectomized animals.

15. Threshold oestrogenic stimulation did not lead to an increase in the size of the adrenals of hypophysectomized rats.

16. It is concluded that the adrenal glands fluctuate in size in a period corresponding to the oestrous cycle. The changes are independent of either the anterior lobe of the pituitary or the gonads, and in some way, as yet undetermined, they exercise a direct effect on the accessory reproductive organs.

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THE EFFECT OF DESOXYCORTICOSTERONE ON THE ENDOMETRIUM OF MONKEYS

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THE fact that the properties of progesterone and desoxycorticosterone overlap is indicated on the one hand by experiments which show that progesterone produces some of the specific effects of desoxycorticosterone and on the other by experiments which demonstrate that desoxycorticosterone has a progestational effect on the endometrium. In the first case it has been shown: (a) that progesterone prolongs the life of adrenalectomized animals [Gaunt & Hays, 1938, working on ferrets; Gaunt, Nelson & Loomis, 1938; Bourne, 1939; Schwabe & Emery, 1939; Tobin, 1939; Greene, Wells, & Ivy, 1939, all working on rats; Corey, 1939, working on cats], an observation which explains the longer survival time of animals adrenalectomized in pregnancy or pseudo-pregnancy [e.g. Rogoff & Stewart, 1927; Swingle, Parkins, Taylor, Hays & Morrell, 1937; Gaunt & Hays, 1938] and of adrenalectomized animals treated with luteinizing hormone [D'Amour & D'Amour, 1939]; (b) that progesterone causes water and salt retention [Thorn, Nelson, & Thorn, 1938]; and (c) that progesterone, like cortical extracts, leads to cortical involution [Clausen, 1940]; progesterone, however, unlike cortical hormone, does not affect the work-capacity of adrenalectomized dogs and rats [Waterman, Danby, Gaarenstroom, Spanhoff & Uyldert, 1939; Ingle, 1940]. In the case of the progestational properties of cortical hormone it has been shown that desoxycorticosterone acetate produces a progestational reaction in the uterus of the rabbit in doses of 5–10 mg. (i.e. 5 to 10 times as much as the required amount of progesterone) [Miescher, Fischer & Tschopp, 1938; de Fremery & Spanhoff, 1939; Robson, 1939; Heuverswyn, Collins, Williams, & Gardner, 1939], and in the cat [Leathem & Crafts, 1940]. Like progesterone, desoxycorticosterone is also able (a) to maintain pregnancy in spayed mice and in spayed and hypophysectomized rabbits, (b) to suppress the response of the rabbit uterus to pituitrin, (c) to suppress the oestrous cycle in mice and (d) to inhibit the effects of oestrone [Robson, 1939]. Desoxycorticosterone has also been claimed to be weakly oestrogenic [Heuverswyn, *et al.*, 1939; Salmon, 1939], and androgenic [Hooker & Collins, 1940]; the latter conclusion, however, is denied by Greene & Burrill [1940].

In an earlier study [Zuckerman, 1939] it was reported that large amounts of commercial cortical extracts were unable to retain in the shaved skin of

pig-tailed monkeys (*Macaca nemestrina*) water that had deposited there as a result of oestrogenic stimulation or, in five experiments, to inhibit or delay the uterine bleeding that normally follows the cessation of oestrogenic stimulation. The latter finding implied (although it did not prove) that the cortin had failed to induce a progestational reaction in the uterus. The following ten experiments were therefore carried out with desoxycorticosterone acetate to test this point, and to discover whether, like the extract, the crystalline hormone is unable to delay oestrogen-withdrawal uterine bleeding.

EXPERIMENTAL

Five mature spayed monkeys (*M. mulatta*) were used in these experiments, details of which are given in Table I. All injections were made intramuscularly in oil solution. Daily vaginal lavages were made to determine the occurrence of uterine bleeding.

In each experiment oestrone was given daily for 14 days in the amounts indicated in Table I. From the 15th day onwards until uterine bleeding occurred, desoxycorticosterone acetate was given daily. In four experiments, 422.21, 419.19, 217.16 and 427.27, the injections of desoxycorticosterone were not continued until oestrone bleeding occurred. In the first

Table I. *The occurrence and inhibition of post-oestrogen bleeding after desoxycorticosterone injections*

Exp. No.	Oestrone given days 1-14		Desoxycorticosterone given from day 15 onwards			Result	Duration of bleeding (days)
	Daily (µg.)	Total (mg.)	Daily (mg.)	Days	Total (mg.)		
419.17	50-300	2.7	5	11	55	Bleeding on 26th day	5
217.15	10-200	1.3	5	11	55	" " 26th "	5
422.18	20-100	1.04	5	11	55	" " 26th "	6
422.19	20-100	1.04	10	13	130	" " 28th "	5
422.21	20-100	0.78	40	28	1120	Hysterectomy 43rd day	—
419.19	20-50	0.58	10-20	9	140	Autopsied on 24th day	—
217.16	20-50	0.58	20	27	540	Bleeding and hysterectomy on 41st day	—
427.26	10-25	0.27	10	11	110	Bleeding on 26th day	6
429.23	10-25	0.27	10	14	140	" " 29th "	6
427.27	10-25	0.27	15	26	390	No bleeding by 40th day	—

and third of these four exceptional cases a sub-total hysterectomy was performed, under nembutal anaesthesia, on the 43rd and 41st days of the experiment respectively. In the second exceptional case the animal was autopsied on the 24th day. In the fourth injections were discontinued on the 41st day.

The specimens of uterus obtained in this way were sectioned at various levels and stained with iron haematoxylin and eosin, haemalum and eosin,

iron haematoxylin and Van Gieson's stain, and by Masson's iron-haematoxylin-ponceau-light-green stain.

RESULTS

The interval before uterine bleeding in spayed rhesus monkeys given amounts of oestrone similar to those administered in the present experiments does not vary significantly (means between 6.5 and 11.5 days [Zuckerman, 1937]). It will be seen from Table I that the occurrence of uterine bleeding was not significantly delayed in any of the animals given 5 or 10 mg. of desoxycorticosterone daily from the 15th day of the experiment onwards. This result agrees with that of the experiments carried out with cortical extracts. From these experiments it is apparent that even when the total amount of desoxycorticosterone given is more than 100 times that of the oestrone administered, the former is unable to prevent the uterine bleeding that normally occurs after cessation of oestrogenic stimulation.

In three cases, experiments 427.27, 217.16 and 422.21, larger doses of desoxycorticosterone were given, in the first 15 mg. daily, in the second 20 and in the third 40. In all three the occurrence of uterine bleeding was significantly delayed. The animal used in the second of these two experiments was hysterectomized on the 41st day of the experiment. Microscopic examination (see below) shows that its uterus had been removed on the first day of uterine bleeding. In the third experiment the animal's uterus was removed on the 43rd day of the experiment. The endometrium, as is also shown below, had undergone progestational differentiation and showed no sign of breakdown.

Three specimens were available for histological examination. The first (419.19) was removed on the 24th day of the experiment after the animal had been given a total of 140 mg. of desoxycorticosterone acetate over a period of 9 days. Figs. 1, 2 and 3 (Plate I) show the remarkable appearance of its endometrium. Most of the glands are more dilated and much more tortuous than is usual in animals that have been given oestrogenic hormone alone. The stromal cells are more or less uniformly packed throughout the endometrium, some are in process of division, and there is relatively little oedema.

The surface epithelium is composed of medium-sized, heaped columnar cells, the free margins of which are covered with globules of secretion. Their ciliae are not distinct.

The glandular epithelium is typically tall columnar, the long oval nuclei occupying about half of the cells, which are usually ciliated. Numerous mitotic figures are to be observed in the glandular epithelium.

The abnormal features of the endometrium of this specimen are as follows.

In several places the lining of the glands has become folded and invaginated into the lumen or evaginated from their mouths in the form of long processes extending into the cavum uteri. In some an invaginated fold of epithelium appears as an epithelial tube lying within the main lumen of a gland, and in others 'invaginations' have occurred into the 'secondary included gland' to form yet another apparent tube within the original gland lumen (see Plate I, Figs. 2 and 3). In these cases the lining cells of the inner tubes appear to be completely healthy and are ciliated. Their only obvious differences from the basal cells of the glands in which they occur is that they usually stain more deeply and that they are more tightly packed.

The evaginations from the mouths of the glands into the cavum uteri (Plate I, Fig. 1) take the form of tongue-like growths in which the epithelium is folded in a complex way. In some cases the projections, which consist entirely of epithelial cells, appear as glands within glands. In other cases the folding of the epithelium of the glands within the endometrium takes an even more complex form.

The cavum uteri is crypto-bicornuate, the main lumen dividing into two in the fundus. The glandular folding occurs in the upper part of the body of the uterus and extends into one of the horns; it is absent in other parts of the uterus. It is also completely restricted to the posterior part of the endometrium.

The second specimen (217.16), which was removed from an animal that had been injected for 27 days with 20 mg. desoxycorticosterone acetate daily, has an entirely different structure (Plate II, Figs. 4, 5, 6). The endometrium is deep and its glands consist of narrow tubules identical with those which are characteristic of the interval (oestrogen) endometrium. The glands are fewer in number than in the previous specimen and are tortuous only in their deepest parts. None shows any sign of progestational differentiation. The endometrium is in an early phase of interval bleeding.

The stroma is denser towards the cavum uteri and is somewhat oedematous in its deeper parts. There are very few mitoses in the stromal cells.

The surface epithelium consists of a single layer of regularly disposed, medium-sized columnar cells, whose oval nuclei occupy the greater part of the cells. There are numerous mitoses in these cells, and mitoses are also to be seen in shed epithelial cells. The stroma beneath the surface epithelium is disorganized by large lakes of blood, which in places have disrupted the surface epithelium.

The glandular epithelium consists of taller columnar cells with basally disposed oval nuclei. The free borders of the cells are usually covered with droplets of secretion, and in some places they are ciliated. Numerous mitotic figures occur in the glands.

The third specimen (422.21) was removed for microscopic examination from an animal which had been injected for 28 days with 40 mg. of desoxycorticosterone acetate daily. The structure of the endometrium is completely different from that of the other two specimens and in places demonstrates a typical progestational reaction (Plate III, Figs. 7, 8, 9). Thus the glands to both lateral aspects of the cavum uteri are widely dilated, tortuous and sacculated. The epithelium of the sacculated glands has in most places undergone a typical progestational change. The nuclei of the cells are relatively small and basally disposed, and their more superficial cytoplasm is clear and vacuolated. They differ from typical progestational cells in so far as their free borders are clearly defined, and not in the form of fringe-like processes of secretion pointing into the lumina. In many glands cellular differentiation has proceeded irregularly and a patch of progestational cells is found lying adjacent to a patch which is still in a condition characteristic of oestrogenic stimulation. The glands immediately anterior and posterior to the cavum uteri are mostly straight, but they are more dilated than is usual in an interval endometrium.

The surface epithelium consists of irregularly arranged low columnar epithelium, whose basally disposed nuclei fill the greater part of the cells, a few of which are in mitotic division. Patches of these cells are also encountered which have undergone progestational secretory differentiation.

The epithelium of the straight posterior and anterior glands is mostly in a typical interval condition; a few mitoses were observed in these cells.

The stroma is very oedematous and vascular, dilated capillaries occurring in many regions and particularly in the superficial zone. In addition to dilated vessels, numerous spaces are found in the superficial stroma filled with polymorphs and large monocytes. Other very large cells which appear in these spaces have non-staining cytoplasm, and round nuclei which occasionally have an eosinophilic patch adjacent to them. Occasionally, too, red blood-cells are found in these spaces, a finding which suggests that these collections of blood-cells indicate an inhibited phase of uterine breakdown.

SUMMARY

Desoxycorticosterone acetate, in daily injections of 5 or 10 mg., is unable, like commercial extracts of cortical hormone, to inhibit or delay oestrogen-withdrawal uterine bleeding in mature spayed monkeys. In larger amounts (15, 20 or 40 mg. daily) uterine bleeding is inhibited. Of the three specimens removed after treatment with desoxycorticosterone only one showed a typical progestational reaction. The animal from which this specimen was taken had been given 1120 mg. of desoxycorticosterone. In the second the uterus was in an interval condition in spite of the admini-

stration of 540 mg. of cortical hormone. In the third, which was given 140 mg. of desoxycorticosterone, the endometrium was abnormal. Many of the glands were more tortuous than usual, and in one region of the uterus the epithelium of almost every gland had invaginated into, or evaginated out of, the individual glands.

A similar transformation of the endometrium has not been observed in any one of the several hundred experimental rhesus monkeys which have been studied in this laboratory.

For the hormones used in these experiments I am much indebted to Dr. K. Miescher of the Ciba Company. The animals were bought with the aid of a grant from the Medical Research Council and the work was also supported by a grant from the Nuffield Medical Committee, Oxford.

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NOTE ADDED IN PROOF

In a recent paper Speert [1940] describes three experiments which suggest that desoxycorticosterone acetate is oestrogenic in the rhesus monkey, in so far as it may increase vaginal desquamation, cause sexual-skin coloration and stimulate lobule-alveolar growth in the mammary gland.

Speert, H. [1940]. *Bull. Johns Hopkins Hosp.* **57**, 189.

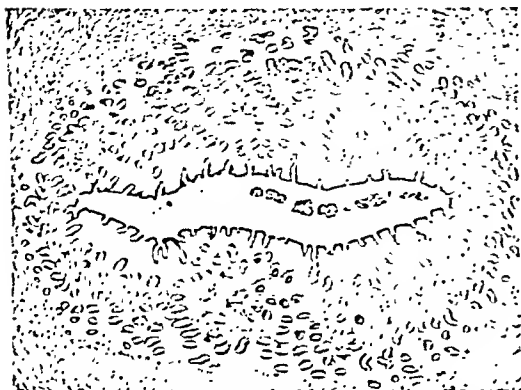


FIG. 1. Low-power view ($\times 10$) of endometrium of 419.19. Cross-sections of epithelial outgrowths from the glands are to be seen in the cavum uteri.

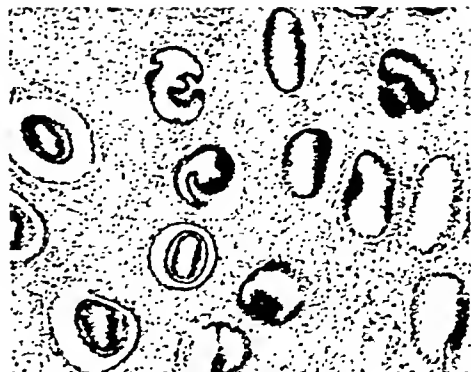


FIG. 2. Eccentric folding of glandular epithelium on posterior part of endometrium of 419.19. ($\times 64$)

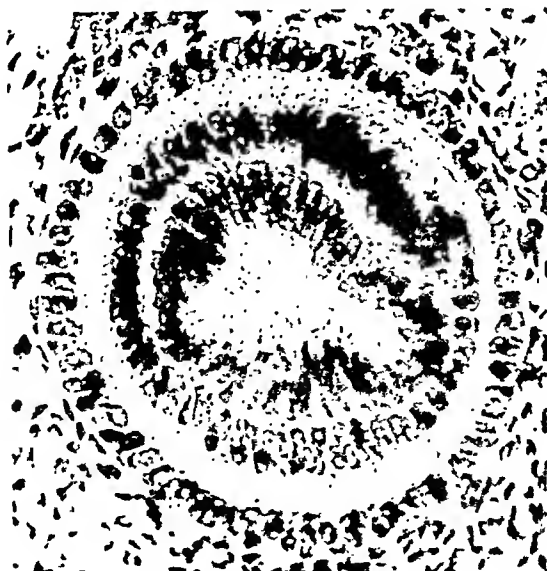


FIG. 3. High-power view ($\times 456$) of endometrium of 419.19. Epithelial lining has invaginated into the lumen to form two concentric layers of cells. The outer cells are basally nucleated.



FIG. 4. Low-power view ($\times 10$) of endometrium of monkey (217.16) which had been given 540 mg. of desoxycorticosterone acetate. The endometrium is in a typical interval condition and in an early phase of bleeding.



FIG. 5. Higher-power view of 217.16 ($\times 78$) showing lakes of blood in the superficial zone of the endometrium.

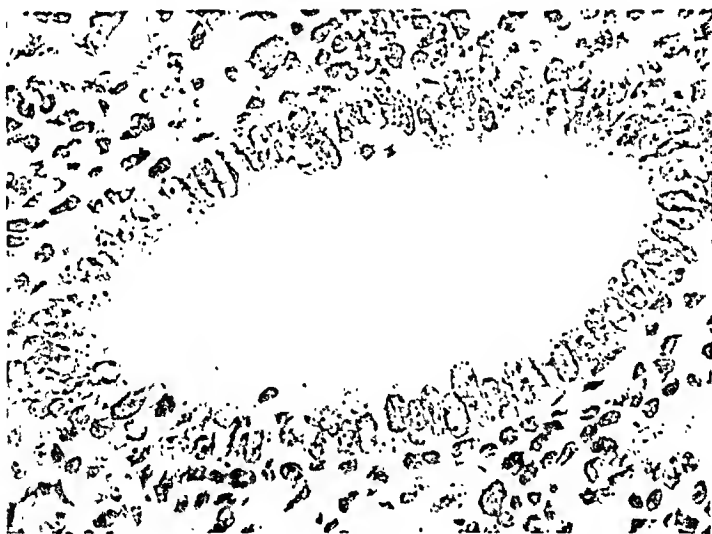


FIG. 6. High-power view of gland in 217.16 ($\times 530$) showing typical interval condition and two mitotic figures.



FIG. 7. Lateral aspect of endometrium of 422.21, which had been given 1,120 mg. of desoxycorticosterone acetate. The glands show a typical progestational reaction. $\times 32$

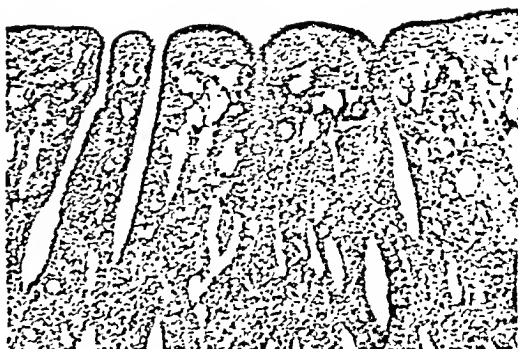
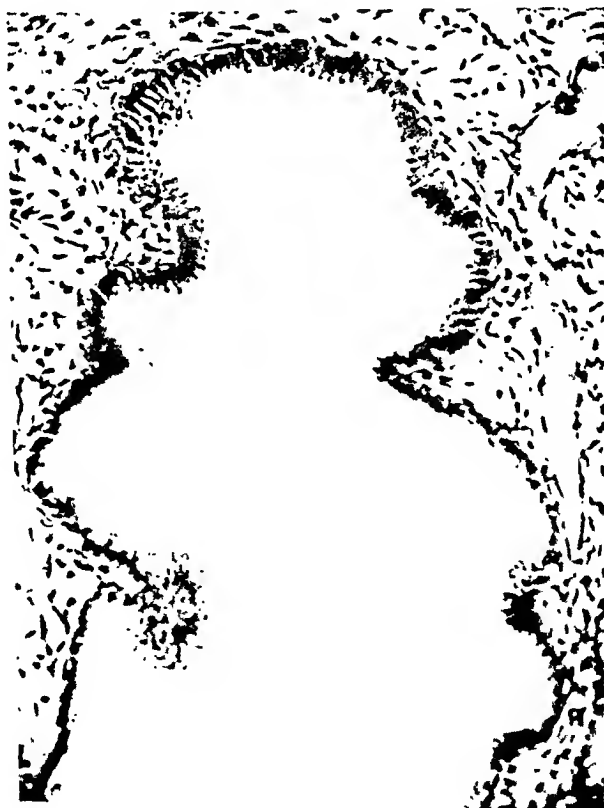


FIG. 8. Undifferentiated tubular glands in same specimen (422.21) on anterior surface of cavum uteri. The stroma is very vascular and the spaces in the surface endometrium are the blood lakes referred to in the text. $\times 60$



THE INFLUENCE OF THE PLACENTA ON THE CORPUS LUTEUM OF PREGNANCY IN THE MOUSE

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EXPERIMENTAL studies of pregnancy in laboratory animals have shown that if the placentae are left attached to the uterus while all the foetuses are removed, the functional activity and histological structure of the corpora lutea are maintained. Klein [1933] performed the operation on the rabbit and repeated it subsequently on the rat [Klein, 1935*a*, *b*] and on the golden hamster [Klein, 1938]. Similar experiments in the rat were carried out by Selye, Collip & Thomson [1935*b*] and certain results of placental retention in the mouse were described by Newton [1935]. Newton & Lits [1938] showed that most of these results necessitated the simultaneous presence of the ovaries. Newton & Beck [1939] have since shown that the pituitary gland can be removed without materially affecting the results, and consequently a relationship in which the pituitary is not concerned exists between the placentae and the ovaries. Hypophysectomy, unlike ovariectomy, commonly does not precipitate abortion either of the total products of gestation or of the retained placentae in the mouse.

The present note describes experiments in which a particular study was made of the corpora lutea of pregnancy in relation to the retention or otherwise of the placentae after foetal destruction.

EXPERIMENTAL

The majority of the mice were hypophysectomized on the 12th day of pregnancy and killed on the 19th day. The method employed for hypophysectomy was that of Thomas [1938] which effects complete removal except for a fragment of stalk where it fuses with the hypothalamus. This fragment carries a layer of cells of the pars tuberalis [W. H. Newton & K. C. Richardson, 1941]. There is no damage to the hypothalamus, but accidental injury is sometimes done to the medulla without any apparent effect on the animal.

Foetal destruction, by rupture of amnion [Newton, 1935] was carried out on the 12th day of pregnancy and in 9 hypophysectomized animals the placentae were retained till the time for killing. In 7 hypophysectomized animals detachment of the placenta from the uterine wall on the 12th day,

or abortion between then and the 17th day, provided a series of mice lacking placentae for various intervals up to a week before killing. The ovaries were fixed one in Bonin's and the other in Flemming's fluid. The corpora lutea were compared as to size by making serial sections of the ovary and taking the mean of the two largest diameters perpendicular to each other of one or more of the largest corpora lutea. Sections were also cut from the ovaries of normal control animals 12 and 18 days pregnant respectively, and from non-hypophysectomized animals subjected to foetal destruction on the 12th day and carrying retained placentae till the day of autopsy.

RESULTS

Table I shows that there was an obvious difference in the size of the corpora lutea between the mice with retained placentae and those without

Table I

Serial No. 390	Condition of mouse							Diam. corpus luteum (μ)
(1) <i>Normal and non-hypophysectomized</i>								
510A	Normal 12th day pregnancy							820
515A	"	18th	"	"	"	"	"	960
515B	"	18th	"	"	"	"	"	916
511D	Foetal destruction 12th day pregnancy; killed 18th day							1064
511G	"	"	12th	"	"	"	18th "	1008
(2) <i>Hypophysectomized, with retained placentae</i>								
111B	(G)	Foetal destruction and hypophysectomy 12th day; killed 19th day						952
112D	(I)	"	"	"	12th	"	" 19th "	916
117A	(J)	"	"	"	12th	"	" 19th "	952
117B	(K)	"	"	"	12th	"	" 19th "	900
117C	(L)	"	"	"	12th	"	" 19th "	880
124C	(X)	"	"	"	12th	"	" 19th "	896
516A	"	"	"	"	12th	"	" 18th "	1008
614A	"	"	"	"	12th	"	" 18th "	1008
621A	"	"	"	"	12th	"	" 18th "	940
							Average (9 mice)	939
(3) <i>Hypophysectomized; placentae removed or expelled</i>								
112B	(H)	F.D. and hypo. 12th day; abortion during 17th; killed 19th						720
116A	(O)	"	"	12th	"	"	16th; " 19th	602
124B	(R)	"	"	12th	"	"	14th; " 19th	592
117D	(P)	"	"	12th	"	"	13th; " 19th	528
124A	(Q)	"	"	12th	"	"	13th; " 19th	536
119A	(S)	Uterus evacuated 12th day; hypophysectomy 13th; killed 19th						472
122A	(T)	"	"	12th	"	"	13th; " 19th	576
							Average (7 mice)	575

The letters in brackets after some of the serial numbers refer to the designation given to the same mice by Newton & Beek [1939].

placentae. In the former group the corpora lutea were as large as in unoperated 18-day pregnant mice, and hypophysectomy had not interfered with their normal growth in the second half of pregnancy. In section they showed no signs of premature regression. In the other hypophysectomized mice, however, similar in all respects except for the absence of placentae, all the corpora lutea of pregnancy were obviously shrunk and showed the usual histological signs of regression (Plate I, Figs. 1-3).

The number of placentae present in groups (2) and (3) ranged from 1-9, but no constant correlation was found between their number and the size of the corpora lutea. It may be noted, however, that in the three mice of group (3) which retained their placentae until the 14th, 16th and 17th days respectively the corpora lutea were progressively larger than the average of the four remaining mice whose uteri were emptied before the 14th day.

DISCUSSION

These experiments show conclusively that the placentae in the absence of the pituitary gland can maintain the growth and activity of the corpora lutea of pregnancy presumably through an endocrine secretion. The problem of whether this resembles one of the known sex hormones or gonadotrophic secretions has already been considered by various writers without any definite conclusions being established. Of the ovarian hormones, progesterone can almost certainly be eliminated as the cause of maintenance of the corpora lutea, although there is some evidence that this substance or a similar one may be secreted by the placenta [Selye, Collip & Thomson, 1935*b*; Haterius, 1936; McKeown & Zuckerman, 1938].

Experiments suggest that the effect of progesterone on the ovary is often inhibitory; it causes the disappearance of corpora lutea from adult non-pregnant mice [Selye, 1939] and it does not maintain the corpora lutea in rabbits hypophysectomized in the first half of pregnancy [Robson, 1937*a*]. On the other hand, definite evidence is available that oestrogens can both enlarge and maintain existing corpora lutea. Hohlweg [1934], Wolfe [1935], and Selye, Collip & Thomson [1935*a*] all found that oestrogen injections caused the production of enlarged corpora lutea, similar to those of pregnancy in the non-pregnant rat. Allen & Heckel [1936] found that oestrogens prolonged the functional existence of corpora lutea of pseudo-pregnancy in the rabbit, and Westman & Jacobsen [1937] and Robson [1937*b*] confirmed this finding in the hypophysectomized pseudo-pregnant rabbit. Later Heckel & Allen [1939] showed that fairly large doses of oestrogen injected into the rabbit towards the end of pregnancy caused immediate death of the fetuses and degenerative changes in the placentae but maintained the corpora lutea of pregnancy and so inhibited parturition. In the light of these experiments and those of other workers, Allen & Heckel

consider that the placenta causes the corpora lutea to persist through its production of oestrogenic hormones. Selye and his collaborators believe that their experiments show that relatively large quantities of oestrogens circulate during the second half of pregnancy in the rat [Selye, Harlow & McKeown, 1935], but they attribute the maintenance of corpora lutea of pregnancy to placental gonadotrophic hormone. In the pregnant mare, however, the appearance of gonadotrophic hormone in the serum coincides with the production of fresh corpora lutea of thecal origin, and the original corpus luteum of pregnancy disappears long before parturition [Cole, Howell & Hart, 1931; Kimura & Lyons, 1937]. Experimental work with known gonadotrophic hormones, moreover, indicates that they commonly have an adverse effect on existing corpora lutea either directly or through their activity in stimulating the formation of new ones [Bunde & Greep, 1936; Greep, 1938]. Furthermore, Selye, Collip & Thomson [1934] call attention to the significant fact that whereas after hypophysectomy thecal deficiency cells in rat ovaries can be prevented from appearing by the known gonadotrophic hormones, yet these cells are seen after hypophysectomy in pregnancy in spite of whatever secretion maintains the corpora lutea. They therefore conclude that in rats the gonadotrophic hormone of the placenta is different from the known gonadotrophic hormones. This conclusion was reached independently by Astwood & Greep [1938] who produced an extract from rat placentae which maintained the function of the corpora lutea of the pseudo-pregnant rat after hypophysectomy, but gave negative results in all the usual sex hormone tests.

Obviously the experiments recorded above throw only a partial light on placental activity. In mice some of the results of placental secretions depend on the presence of the ovaries and probably on functional corpora lutea (e.g. suppression of oestrus, mucification of the vagina, and parturition at term [Newton & Lits, 1938]), while others, such as the later stages of mammary gland development, can be produced independently of the ovaries or pituitary gland [Newton & Lits, 1938; Newton & Beck, 1939]. Gardner [1936] has shown that ligamentous transformation of the symphysis pubis, a placental effect for which ovaries are also necessary, can be effected by oestrogens, and, though the speed with which this occurs in pregnancy indicates that a second factor is involved, it is not yet certain what this may be. Maintenance of an abnormal body-weight [Newton, 1935; Brooksby & Newton, 1938] is another placental effect which has not yet been correlated with luteal activity.

SUMMARY

If the placentae are retained, the corpora lutea of pregnancy in the mouse show no reduction in size or cessation of normal growth following hypo-

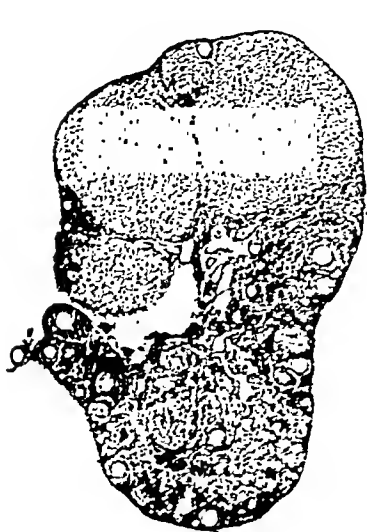


FIG. 1. No. 124 B. Abortion of placentae 14th day; killed 19th day; corpora lutea shrunk.



FIG. 2. No. 112 B. Abortion of placentae 17th day; killed 19th day; corpora lutea have just begun to regress.

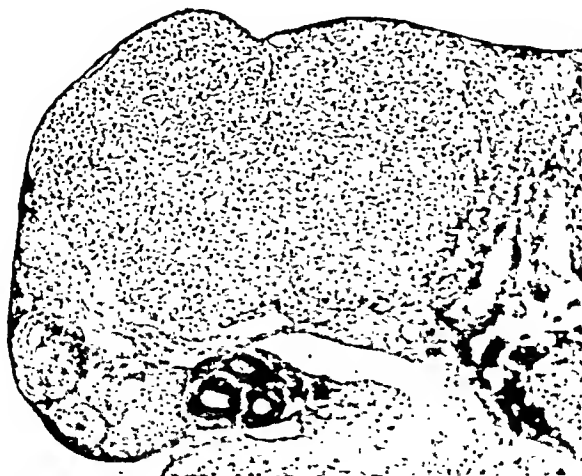


FIG. 3. No. 614 A. No abortion; placentae retained; killed 19th day; corpora lutea as in normal late pregnancy.

physectomy on the 12th day of pregnancy and destruction of the fetuses. Elimination of the placenta causes the corpora lutea to degenerate whether or not the pituitary gland is present. It is clear therefore that anterior pituitary gland secretions are not concerned in the maintenance of mouse corpora lutea in the second half of pregnancy.

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THE SECRETION OF MILK IN HYPOPHYSECTOMIZED PREGNANT MICE

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NEWTON & LITS [1938] showed that the mammary glands of mice attained full development by the end of pregnancy, in spite of removal of foetuses and ovaries a week previously. Newton & Beck [1939] demonstrated continued mammary development after removal, at a similar time, of foetuses and pituitary gland. The retained placenta, the only common factor in these experiments, was therefore established as an important stimulus to late mammary development. The only obstacle to regarding it as the sole stimulus was the fact that in the hypophysectomized series, although all the glands contained a certain amount of secretion, only a few were engorged with milk to the point of alveolar distension. This raised the important possibility that while the placenta might be responsible for growth, the pituitary might be necessary for secretion, and that the vigorous nature of the latter in a limited number of the series might be due to incomplete hypophysectomy. The heads of two mice had been serially sectioned through the operative field, but they had shown only moderate mammary secretion and complete absence of the pituitary was to have been expected. Too late to be reported in the previous paper, Miss Beck confirmed complete absence of anterior lobe tissue in a mouse showing full mammary secretion.

The object of the present investigation was to establish the completeness of hypophysectomy by Thomas's [1938] method, and to obtain additional information about mammary development during pregnancy. Seven mice were hypophysectomized on the 12th day of pregnancy, killed on the 19th or 20th day, the contents of the uterus not having been molested, and the field of operation and the mammary glands examined. A further 5 mice were hypophysectomized on the 12th day of pregnancy and allowed to litter. They were killed as soon as possible after their litters had died, which was within 36 hours of parturition, and the state of their mammary glands compared with that of those in the previous series. Their pituitary regions were not examined.

HISTOLOGICAL METHODS

After removal of the mandible and portion of the parietal bones, the heads of the hypophysectomized mice were placed in alcohol-formalin-

acetic acid fixative (Bodian) for 24 hours, and then transferred to 1% nitric acid until decalcified. A small block was cut from each specimen containing that portion of the brain and the skull base medial to the trigeminal nerves and bounded rostrally by the optic chiasma and caudally by the rostral end of the medulla. The material was placed in 5% sodium sulphate solution overnight and dehydrated in glycerine from which it was transferred directly to a solution of nitrocellulose (Necol) of approximately the same viscosity as the glycerine. Four days' infiltration was found to be sufficient for these specimens. The final imbedding in 25% Necol was carried out in a paper boat containing a small glass plate on which the specimens were arranged side by side to form a single block. In this way the pituitary regions from as many as 12 mice can be cut serially in a single block. The sections, cut at 15μ , were mounted with the collodion intact to ensure that any loose fragments of glandular tissue did not fall from the sections during staining.

The mammary glands were dissected out and pinned on cork before fixation in Zenker-formol (Helly). A portion of each fixed gland was set aside for fat staining by the osmic acid technique of Hoerr [1931].

EXPERIMENTAL RESULTS

Anatomical results of hypophysectomy

Fig. 1 shows a numbered series of coronal sections through the pituitary gland of a normal female mouse. The sections are presented in outline only (drawn with the aid of a projector) to show the size of the whole gland and the relations of its parts. They are 150μ apart. Fig. 2 shows a sagittal section through the approximate centre of a normal pituitary gland outlined at the same magnification. The stalk is recognizable, and carries a layer of tuberalis cells for some distance rostral to its fusion with the tuber cinereum, and is visible 150μ anterior to the first section of Fig. 1.

In the hypophysectomized mice a variable amount of stalk and posterior lobe was left, and was fairly accurately assessed by counting caudally from the first section showing fusion of stalk and brain (corresponding to section 4 of Fig. 1) to the last section showing any trace of pituitary tissue. Table I shows that this was between 240μ and 460μ behind the point of fusion. Figs. 1 and 2 show that a large part of all divisions of the gland would be present at this level had the plane of severance been clean and perpendicular. For contrast, the actual outline of the residua, 100μ rostral to their vanishing point, are shown at the same magnification in Fig. 3. (These shreds diminished in size and became vague in outline caudal to the section traced.) The tracings prove that the glands have been torn away along a line such as that superimposed on Fig. 2. With one exception, no trace of pars anterior or pars intermedia was found in any of the

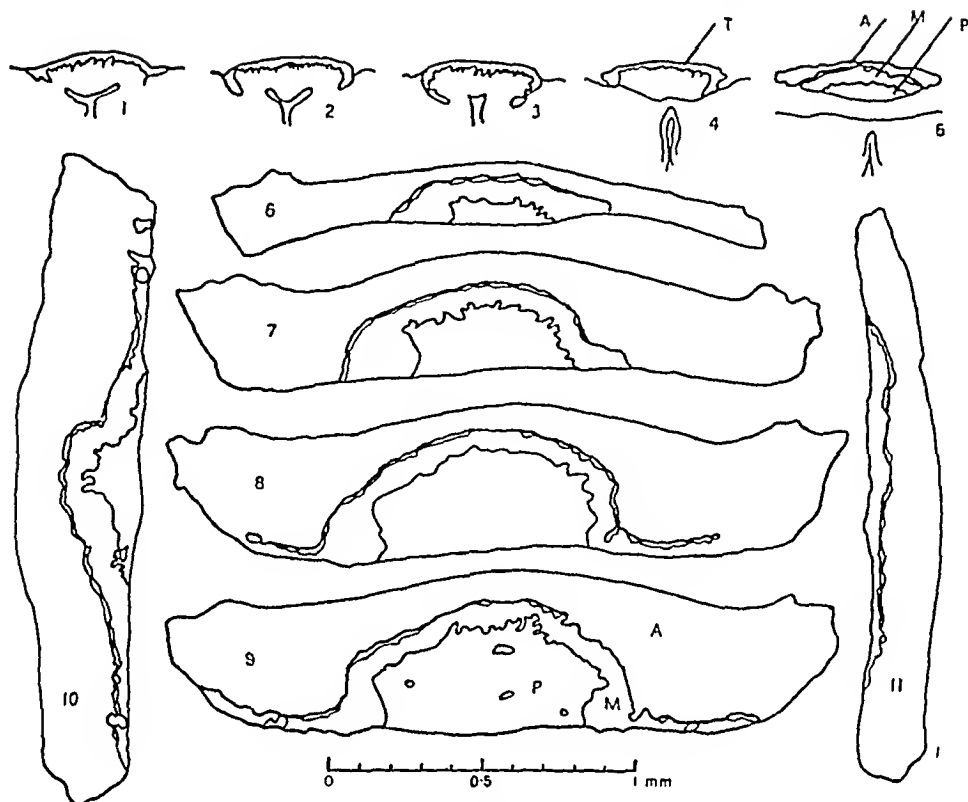


FIG. 1. Eleven coronal sections, 150μ apart, through the pituitary region of a normal mouse comparable in size to those used in the experiments described.

Sequence runs caudally according to numerals. 15μ caudal to section 4, the stalk was no longer fused with the hypothalamus.

Posterior lobe has disappeared in section 11; further sections omitted.

A—pars anterior; M—pars intermedia;
P—pars nervosa; T—pars tuberalis.

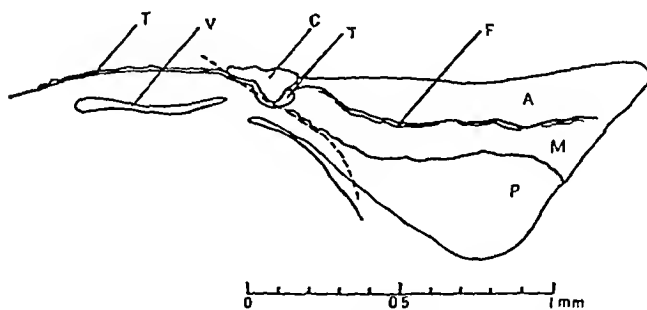


FIG. 2. Sagittal section approximately through the centre of the pituitary gland of a normal mouse, comparable in size to those used in the experiments.

A—pars anterior; C—cyst in pars tuberalis;
F—hypophyseal cleft; M—pars intermedia;
P—pars nervosa; T—pars tuberalis;
V—cavity of 3rd ventricle.

The interrupted line indicates the plane of rupture in Thomas's hypophysectomy. The scale begins for convenience beneath the junction of stalk and hypothalamus.

explanation of the direction taken by the plane of rupture is probably to be found in the relatively fibrous structure of the pars nervosa.

The exception, 1212A (Fig. 3), showed the hypophyseal cleft, the inferior surface of which was outlined by a layer of cells which must belong to the

Table I

Serial No. 39	Length post-pit. remnant (μ)	Serial No. 400	Length post-pit. remnant (μ)
1211A	300	113A	260
1211B	240	116A	320
1211C	360	124A	380
1212A	460		

Average length=334 μ ; omitting 1212A=313 μ .

pars anterior; its superior surface was formed by little more than a single layer of pars intermedia cells, representing an enormous shrinkage of this part of the gland (cf. Fig. 1), possibly due to lack of blood supply. The doubtful vitality and small size of this fragment, rough calculation sug-

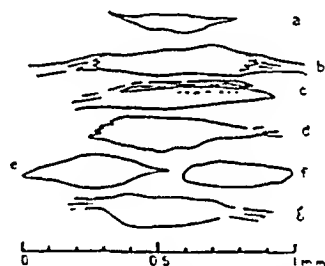


FIG. 3. Coronal sections through pituitary remnants 100 μ rostral to their point of disappearance.

a—1211 C; b—1211 B; c—1212 A; d—1211 A; e—113 A; f—116 A; g—121 A.

Only c contains anything besides posterior lobe tissue.

gesting that it may represent 1% of the original total anterior lobe tissue, probably render it functionally negligible.

The 3 operations mentioned as having been previously tested by serial sections bring the total number studied up to 10, and this warrants the conclusion that Thomas's method of hypophysectomy is reliable.

The state of the mammary glands

The mammary glands of the mice killed on the 19th or 20th day of pregnancy, 7 or 8 days after hypophysectomy, were fully developed and distended with milk (Plate I, Figs. 4 and 5). Five out of the 7 mice were found with litters on the morning of autopsy, and although the stomachs of some of the young contained fluid, milk was found definitely only in the litter of 116A.

The 5 remaining mice hypophysectomized on the 12th day of pregnancy were allowed to litter, and the litters left with them. When they were lifted from the cage the young remained hanging to their nipples, and were obviously suckling vigorously. Nevertheless, no milk was found in the stomachs of young which were occasionally killed for examination (the stomachs could also be observed through the transparent abdominal wall). Only in the litter of one mouse, which had still 3 undelivered foetuses in utero, were the stomachs markedly distended with slightly turbid fluid. Without exception the litters died on the day following that on which they were found, or during the intervening night. No milk was found in their stomachs at autopsy, and at the end of their first day of life they appeared emaciated; members of 3 litters which were weighed had lost 10–15% of their morning weight by 5.0 p.m.

Immediately their litters died, or on the morning following overnight death of the litter, the mice were killed. In contrast to those of the previous series, killed just before or just after littering, the mammary glands of these mice showed a marked degree of involution (Plate I, Fig. 6).

DISCUSSION

Sporadic accounts have previously appeared of a transient lactation after parturition in hypophysectomized animals [see literature cited by Nelson, 1936; Newton & Beck, 1939 and Robson, 1940]. The results just described establish beyond doubt that complete full-term mammary development, including the secretion of milk, occurs in pregnant hypophysectomized mice. In certain instances [see also Newton & Beck] the young may obtain a little milk at first, but this is not a constant finding and they frequently fail to do so in spite of vigorous suckling. We conclude, therefore, that the production of milk ceases after delivery of the placenta, and that suckling alone is insufficient to maintain secretion. The rapid involution of the glands after parturition is in accord with this, and the failure of lactation emphasizes the absence of the pituitary.

Incomplete hypophysectomy having been eliminated as a cause of full secretion in the experiments of Newton & Beck, it follows that although this occurred only in 5 or 6 out of 19 of their cases, the placenta is capable of completing by itself the normal mammary development of the last week of pregnancy. The reason why full secretion did not occur in all their cases, while occurring in every case of the present series, has two possible explanations. First, the foetal endocrines may play a part; secondly, the placental secretion may sometimes be quantitatively insufficient. The only theory which seems to cover the facts is that the placenta, the maternal pituitary and the foetal endocrines all exert similar effects. If either of the last two are present with the placenta [cf. Newton & Lits, 1938] the

mammary glands are developed to the maximum. If the placentae alone are present, they may be insufficient, either through having their development arrested at the 12th day or through trauma at foetal destruction, to bring the mammary glands to the point of full secretion. Other placental effects which have been described are dependent on the simultaneous presence of the ovaries, and may operate through a trophic effect on the ovaries [Deanesley & Newton, 1941]. If this be so, a mass of placental tissue more than sufficient to maintain the ovaries might yet be incapable of a maximal trophic effect on the relatively enormous combined mammary glands. This would explain the degrees of partial secretion found by Newton & Beck in such of their glands as failed to secrete maximally.

Progesterone is very unlikely to be the agent by which the placentae produce their effects on the mammary glands, because the ovaries are unnecessary for this aspect of placental activity. Since placentae have a marked trophic effect on the corpora lutea [Deanesley & Newton, 1941], progesterone from the ovaries is evidently necessary to supplement any produced by the placentae themselves, and several placental effects disappear when the ovaries are removed. Some of these (e.g. mucification of the vagina [Klein, 1937]) are known to require progesterone, which must therefore be assumed to be relatively or totally deficient in the absence of the ovaries. Available evidence, including the non-oestrogenic gonadotrophic extract of Astwood & Greep [1938], is consistent with the placentae having an anterior pituitary-like function, but though artificially administered oestrin is incapable of producing in non-pregnant mice the degree and kind of mammary development found, it may do so in mice already 12 days pregnant, and therefore cannot be excluded.

Owing to the incomplete removal of posterior pituitary tissue, even though the amount left was small, no light is shed by our results on the necessity or otherwise of this part of the gland for parturition [cf. Fisher, Magoun & Ranson, 1938]. The labours of the 10 mice which littered were not observed, though one was known to deliver 8 young within one hour, and another to have retained 3 foetuses in utero for at least 8 hours after the birth of 4 young. Of the total of 58 young found, only 9, all in 3 litters, were found dead; they may not have been born dead.

SUMMARY

Serial sections through the heads of 10 mice (7 in this series and 3 previously) following hypophysectomy by Thomas's method show that the operation is reliable for removing anterior and intermediate lobe tissue. A trace, possibly amounting to 1% of the normal total, of anterior lobe tissue was found in one specimen only. The layer of tubular cells at the

the infundibular part of the stalk cannot be removed, and in addition to this part of the stalk a shred of posterior lobe remains.

In the mouse the placenta is the chief agent determining growth of, and secretion by, the mammary glands during the last week of pregnancy. The anterior lobe of the pituitary gland is necessary for lactation after parturition.

We wish to acknowledge the hospitality accorded to our departments and the facilities given for the continuance of our work by Professor T. Graham Brown and Mr. T. H. Burlend of the University College of South Wales, Cardiff. The expenses of this research were defrayed by a grant from the Medical Research Council to whom our best thanks are due.

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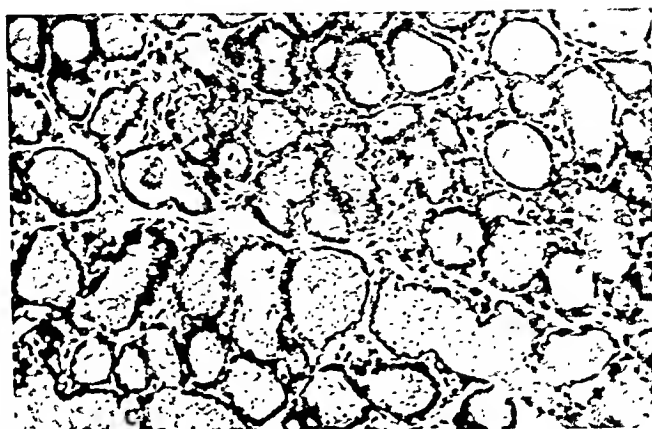


FIG. 4. Section through 4th mammary gland of hypophysectomized pregnant mouse on 20th day of pregnancy. $\times 210$

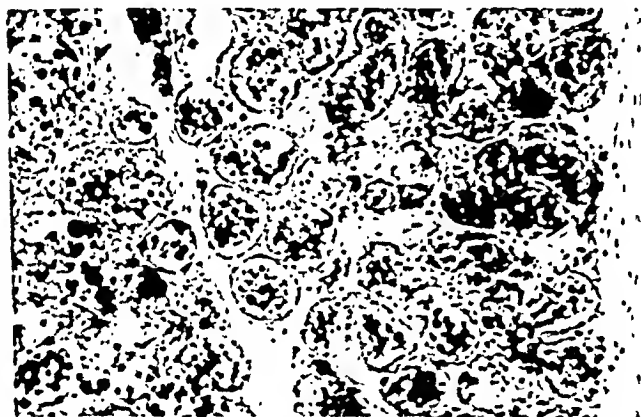


FIG. 5. Portion of same gland as in FIG. 4, stained to demonstrate fat. $\times 210$

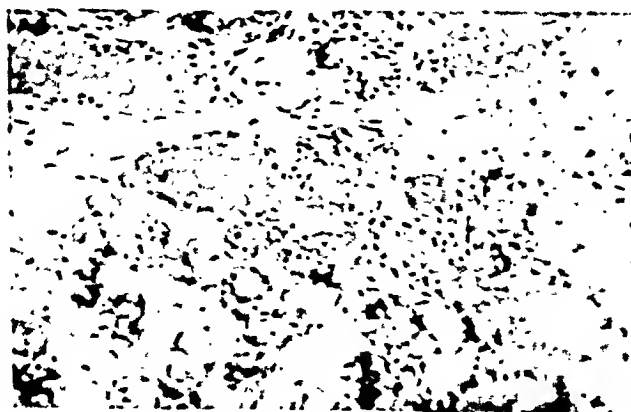


FIG. 6. Portion of same gland as in FIG. 4, stained to demonstrate glycogen. $\times 210$

INFLUENCE OF THE PITUITARY ANTERIOR LOBE UPON THE SPECIFIC DYNAMIC ACTION OF PROTEIN

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THE results obtained in the numerous experiments dealing with the influence of hormones on the specific dynamic action of protein are extremely contradictory; even the simple question as to how the specific dynamic action of protein is influenced by pathological states of hypo- and hyper-thyroidism has not been answered satisfactorily [Serejski & Jisslin, 1930]. Abelin [1923] reports an increase in the specific dynamic action of protein after administration of thyroid extracts, while Feuling [1933] and Schittenhelm & Eisler [1932] observe a decrease after administration of thyrotrophic hormones. The reports on the specific dynamic action in hypophyseal pathology are especially conflicting. Plaut [1922 *a, b*], Liebesny [1925], Kestner, Liebeschütz-Plaut & Schadow [1926] and Peters [1930] found a decreased specific dynamic action of protein in pituitary hypofunction. Fulton & Cushing [1932] and Johnston [1932] were, however, unable to observe any change in patients with disturbed hypophyseal function. Gaebler [1929] found the specific dynamic action of protein unchanged in a hypophysectomized dog. According to Foster & Smith [1926] the specific dynamic action of glycocol is decreased in hypophysectomized rats, though Evans, Luck, Pencharz & Stover [1938] found it unchanged.

The present experiments were carried out in order to study systematically the specific dynamic action of protein in rats at certain times after hypophysectomy and also the influence of some of the well-known hormones of the pituitary anterior lobe on the specific dynamic action.

METHODS

injection needle, on the point of which a piece of thin ureter catheter was fixed. As this could easily be passed into the stomach of the animals, administration was carried out very quickly and irritated the animal only slightly. Immediately after the administration the animal was again put in the metabolism apparatus. The first metabolic measurement was made thirty minutes afterwards. Further measurements followed at hourly intervals as shown in Tables I–III. The changes in oxygen consumption following infusion were studied in about 320 experiments each lasting 6–8 hours.

The apparatus used was a modification of that previously described by Reiss, Kusakabe & Budlowsky [1938]. The animals were enclosed in a bottle of about 1 litre capacity with a perforation at the bottom. The bottle was connected with the air circulation train by airtight connexions and was completely immersed in a thermoregulated water-bath maintained at 28°. At this temperature the animals remained perfectly quiet and slept almost the whole time. Air was circulated through the respiration chamber by means of a pump of the syringe type, worked by a variable eccentric so as to permit variation of the rate of air flow. Unidirectional air flow was secured by a Muller valve of very low resistance. Before entering the respiration chamber, the air was passed through a spiral tube immersed in the water-bath while the air leaving the chamber passed through a soda lime tower in which the carbon dioxide was completely absorbed. The system was connected with a calibrated spirometer with a pointer writing on a smoked drum. The consumption of oxygen in a given time could be calculated from the tracing in the usual manner.

The following anterior pituitary preparations were used in the experiments: (1) a growth hormone extract, prepared by ourselves, which was free from thyrotrophic, corticotrophic and almost free from gonadotrophic hormone; 1 mg. of the dry substance contained one biological unit [Freud, 1935]. As this extract was still relatively crude as compared with extract (2), the possible presence in it of other factors acting on the metabolism cannot be excluded; (2) a growth hormone extract obtained by Dr. Freud, Amsterdam, which was much more purified and contained 1 unit in 6 μ g.; (3) a thyrotrophic extract, completely free from gonadotrophic and corticotrophic hormone and containing 20 guinea-pig units [Junkmann & Schöller, 1932] per mg.; (4) a corticotrophic extract, completely free from thyrotrophic and gonadotrophic hormone, containing 3 units [Reiss, 1936] in 1 mg.; (5) a gonadotrophic extract, obtained from serum of pregnant mares, containing 40 units (oestrus-producing units) in 1 mg.

RESULTS

Table I shows the extent of the specific dynamic action after administration of a certain quantity of protein in normal rats and in rats before

Table I.

Date.	Body-weight (g.)	Experimental details	Oxygen consumption per minute in ml.										
			Before admini- stration	30	60	90	120	150	180	210	240	270	
11 17	304	Untreated	5.80	0.21	5.03	5.80	7.72	0.79	0.02	0.20	5.07	5.20	
	265	6 days after hypophysectomy	5.05	—	5.74	0.37	5.05	0.00	5.85	0.00	5.20	—	
	276	15 days after hypophysectomy	2.80	—	2.69	2.78	3.07	3.07	2.08	3.06	2.07	—	
	227	30 days after hypophysectomy	2.16	2.10	2.03	2.09	1.92	1.87	1.76	1.70	1.82	1.69	
11 14	250	Untreated	4.22	4.07	4.20	5.58	4.89	5.73	4.57	4.37	4.87	4.78	
	220	6 days after hypophysectomy	3.50	3.98	3.67	3.88	3.28	3.68	3.57	3.84	—	—	
	201	30 days after hypophysectomy	2.00	2.47	2.58	2.36	2.28	2.18	2.12	2.16	2.01	1.98	

Table II.

Rat no.	Body-weight (g.)	Experimental details	Oxygen consumption per minute in ml.									
			Before admini- stration	30	60	90	120	150	180	210	240	270
H 115	146	21 days after hypophysectomy	1.86	—	1.63	1.63	1.55	1.67	1.47	1.57	1.57	—
	143	2 days treated with 1 ml. extract inactivated by heat	1.90	—	1.75	1.73	1.65	1.65	1.67	1.83	1.67	1.83
	140	5 days the same treatment	2.10	2.18	2.15	1.80	1.85	1.85	1.80	1.70	1.68	1.60
	140	13 days the same treatment	2.13	2.27	2.10	1.93	1.93	2.17	1.90	1.83	1.83	2.10
	132	44 days after hypophysectomy 5 days after treatment with 20 units thyrotrophic hormone daily	2.77	2.70	2.96	2.60	2.60	2.52	2.86	2.60	2.50	2.52
H 116	142	68 days after hypophysectomy 5 days treated with 15 mg. of crude growth hormone extract daily	1.90	1.97	1.87	1.97	2.03	1.87	1.97	2.03	2.03	1.97
	148	9 days after the same treatment	1.96	2.13	2.23	2.07	2.17	2.37	2.23	2.01	1.90	1.82
	145	24 days after hypophysectomy	1.85	1.97	1.73	1.63	1.63	1.57	1.50	1.63	1.63	1.63
	140	2 days treated with inactivated extract (see above)	1.98	1.95	1.87	1.67	1.57	1.53	1.57	1.68	1.58	1.67
	137	5 days after the same treatment	1.80	1.90	1.73	1.83	1.60	1.60	1.53	1.53	1.53	1.52
H 117	130	13 days after the same treatment	1.93	1.80	1.83	1.77	1.90	1.87	1.87	1.80	1.87	1.92
	117	42 days after hypophysectomy 5 days after treatment with 20 units thyrotrophic hormone daily	2.33	—	2.20	2.20	2.04	2.23	2.10	2.10	1.08	2.03
	112	8 days after the same treatment	2.30	2.40	2.33	2.23	2.33	2.32	2.32	2.30	2.30	2.19
	100	68 days after hypophysectomy	1.79	1.80	1.71	1.70	1.71	1.63	1.80	1.63	1.63	—
	118	9 days treated with 15 mg. crude growth hormone extract daily	1.63	2.43	1.73	1.90	1.80	2.00	1.80	1.77	1.72	1.72

Table II (continued).

Rat no.	Body-weight (g.)	Experimental details	Oxygen consumption per minute in ml.										
			Before admini- stration	Minutes after administration of 1 ml. egg albumen									
				30	60	90	120	150	180	210	240	270	
1174	130	30 days after hypophysectomy	1.90	1.77	1.67	1.57	1.67	1.77	1.67	1.67	1.63	1.83	
	127	7 days treated with inactivated extract (see above)	2.03	2.07	2.00	1.97	1.90	1.62	1.80	1.97	2.07	1.80	
	120	47 days after hypophysectomy 6 days treated with 20 units thyro- trophic hormone daily	2.87	2.81	2.77	2.17	2.50	2.77	2.68	2.68	2.80	2.80	
1175	115	20 days after hypophysectomy	1.72	—	1.80	1.62	1.70	1.83	1.70	1.80	1.71	1.74	
	131	6 days treated with 15 mg. crude growth hormone extract daily	1.78	2.14	1.90	1.93	2.20	2.14	2.00	2.40	2.07	2.02	

Table III.

Rat no.	Body-weight (g.)	Experimental details	Oxygen consumption per minute in ml.											
			Before admini- stration	Minutes after administration of 2 ml. egg albumen										
				30	60	90	120	150	180	210	240	270		
H 34	229	25 days after hypophysectomy	2.18	2.17	2.15	2.17	1.78	2.04	2.04	2.02	2.03	1.99		
	234	After 4 days' treatment with 20 units (Freud) growth hormone daily	2.28	2.46	2.46	2.02	2.34	2.09	1.97	2.47	2.02	2.33		
	242	After the same treatment for 15 days	2.28	2.40	2.64	2.11	2.60	2.03	2.50	2.39	2.27	2.26		
	249	After the same treatment for 24 days	2.20	2.78	—	2.95	2.66	2.43	2.66	2.64	2.64	—		
H 32	192	25 days after hypophysectomy: un- treated	2.41	2.40	2.05	2.29	2.42	2.16	1.92	—	—	—		
	199	After 10 days' treatment with 40 units (Freud) growth hormone daily	1.98	2.27	2.34	2.66	1.82	1.87	1.87	1.78	1.63	1.63		
	206	After 23 days' treatment with 40 units (Freud) growth hormone daily	1.87	1.82	—	2.87	2.73	2.74	1.93	2.01	2.01	—		
	215	34 days after hypophysectomy, after 2 days' treatment with 40 units (Freud) growth hormone daily	2.46	—	2.54	2.93	2.62	—	—	2.52	2.67	2.57		
(see Table I.)	227	After 7 days' treatment with 40 units (Freud) growth hormone daily	2.34	2.73	3.12	2.33	2.24	2.63	2.53	2.23	1.93	2.32		

and at certain periods after hypophysectomy. In untreated rats the oxygen consumption may increase up to 40% above the normal. Six days after hypophysectomy there is still some specific dynamic action present, although it has decreased. Thirty days after hypophysectomy no specific dynamic action was observed after the infusion of egg albumen in any of the hypophysectomized animals (two examples are shown in Table I), in addition to this it was often found that the oxygen consumption was decreased as much as 30% below the initial normal level. Since we have as yet no explanation of this last fact it will not be discussed further in this paper. It is certainly not due to any injury inflicted on the rats during experiments, as the animals survived and were later used for other purposes.

The hypophysectomized animals were first injected with inactivated hypophyseal extracts containing only a very small amount of corticotrophic hormone. The specific dynamic action remained unchanged (Table II). The same rats were then injected with thyrotrophic hormone, and while the oxygen consumption increased considerably, no specific dynamic action could be observed after the administration of egg albumen (Table II). But if the same animals were injected with a strong though still relatively crude growth hormone extract, they showed a marked specific dynamic action a few days after the administration was started (Table II).

The next experiments were carried out with the specially purified growth hormone which we obtained from Dr. Freud of Amsterdam. This extract increased the specific dynamic action of hypophysectomized animals in all experiments (Table III).

Similar experiments with purified corticotrophic hormone and with gonadotrophic hormones gave, in all cases, negative results. Neither of these hormones re-established the specific dynamic action. In one case only, specific dynamic action was increased by 'Ambinone'—an extract containing gonadotrophic as well as thyrotrophic hormone—but we did not succeed in reproducing this effect in further experiments.

DISCUSSION

structure. It should be stated that the conflicting results obtained by Foster & Smith [1926] cannot yet be explained. It is possible, even probable, that the pituitary anterior lobe affects different amino acids in different ways. The question as to which stage in protein metabolism is disturbed cannot be further discussed here. We do not consider it probable that the absorption of protein is greatly disturbed, since in previous experiments we found that the absorption of carbohydrates was not affected by hypophysectomy, while their combustion was increased [Reiss *et al.*, 1938].

Gaebler [1929], however, observed undisturbed specific dynamic action after the administration of meat to a hypophysectomized dog, but his observations were carried out 11–21 days after hypophysectomy while we were only able to observe the disappearance of specific dynamic action of high molecular proteins in our rat experiments 30 days after hypophysectomy. In spite of this, similar experiments should be carried out on dogs on a larger scale with particular attention to the completeness of hypophysectomy.

The experiments make it clear that only those anterior pituitary extracts containing the so-called growth hormone can re-establish the specific dynamic action of proteins after hypophysectomy. The disappearance of specific dynamic action is therefore due to a lack of growth hormone or—if this hormone, as assumed by several writers, is complex—of the 'growth hormone complex'. The possibility of the presence in the growth hormone of a component acting particularly on the protein metabolism should still be considered, but, taking into account other facts about the physiological action of the growth hormone, it does not appear very plausible. The decrease of nitrogen excretion after administration of growth hormone is well established [Teel & Cushing, 1930; Gaebler, 1933, 1935; Lee & Schaffer, 1934; Lee, 1938] and the non-protein nitrogen and free arginine of the blood are also decreased by such treatment [Teel & Watkins, 1929; Reiss, Schwarz & Fleischmann, 1936]. These facts suggest that there is an increase of protein synthesis after administration of growth hormone. But synthesis of protein depends upon previous degradation of externally administered proteins to the simple elements necessary for protein synthesis.

On the basis of the results we are able to characterize the growth hormone more precisely. Growth hormone is present in the pituitary anterior lobe not only of juvenile animals and human beings but also of adult ones, although in adults there is no further growth. It looks as though the growth hormone, on account of its profound influence on protein metabolism, may act in adults purely on processes of regeneration and therefore this hormone might better be characterized as a *regeneration* hormone.

This point of view may also facilitate a clear definition of the diagnostic importance of the specific dynamic action of protein, entitling us to conclude that individuals with disturbed specific dynamic action are not fully capable of regeneration processes. This interpretation might further explain many other observations in various pathological states and so open the way to revision of treatment.

Other experiments showed clearly that thyrotrophic hormone, while greatly increasing the oxygen consumption of hypophysectomized animals, is not able to re-establish specific dynamic action. This result, though at first surprising—as according to the common conception thyroid function is responsible for specific dynamic action—was fully proved by those of our experiments in which the thyroid function of hypophysectomized animals was completely re-established without any return of specific dynamic action. Apparently the influence of the thyroid on specific dynamic action is indirect and due to its influence on the pituitary anterior lobe; a disturbed pituitary anterior lobe secretion is found after thyroidectomy which may thus bring about a disturbed specific dynamic action. In another connexion we have previously suggested this kind of relation between the thyroid and the pituitary anterior lobe. The growth effect of thyroid, for example, is the indirect result of previous influence on the pituitary anterior lobe. While growth can be induced in thyroidectomized animals by administration of growth hormone, no growth can be seen in hypophysectomized animals after administration of thyroid hormone [Smith, 1933; Reiss & Balint, 1934].

Finally, we may consider the numerous contradictory observations on specific dynamic action in various pathological states. The contradictions in hypo- and hyperthyroid states, for example, can be explained if we suppose that the change in specific dynamic action in one or the other direction does not depend directly upon the existence of hypo- or hyperthyroidism, but rather upon the way the anterior pituitary function is affected by the thyroid disturbance. Moreover, it is possible to explain the paradoxical behaviour of specific dynamic action in various disturbances of anterior pituitary lobe function by supposing that hypofunction of the anterior pituitary lobe does not necessarily involve a disturbance of growth hormone production, but may affect only the production of the other anterior pituitary lobe hormones.

SUMMARY

The specific dynamic action can be re-established by administration of extracts containing growth hormone.

Thyrotrophic hormone, whilst increasing oxygen consumption in hypophysectomized animals, does not influence the specific dynamic action of egg albumen.

Corticotrophic and gonadotrophic hormones do not influence specific dynamic action.

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THE EFFECT OF PREVIOUS OESTROGENIC TREATMENT ON THE RESPONSE OF OVARIECTOMIZED MICE TO OESTROGENS

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IN considering the factors which influence the response of animals to oestrogens Marrian & Parkes [1929] were of the opinion that previous dosage was unimportant. Emmens [1939] has recently reconsidered this question and has arrived at similar conclusions.

In contrast with these experimental findings we have observed clinically that prolonged courses of administration of oestrogens in high doses are followed by a diminution in response. The following experiments were devised to examine the behaviour of ovariectomized mice after similar treatment.

MATERIAL AND METHODS

The animals used in the experiments were adult female mice obtained from dealers. Vaginal smears were taken daily by washing the vagina with a small quantity of normal saline contained in a sterile pipette, and were read unstained. Those animals showing regular cycles were ovariectomized. Oestrogenic treatment was begun within 3 days of the operation. Oestrone (Menformon kindly supplied by Organon Ltd.) was used in aqueous solution and each unit was equivalent to 0.1 μ g.

Smears containing large numbers of cornified cells, with either very few or no leucocytes were taken to indicate oestrus. Such smears are in fact indistinguishable from the oestrous smear of the normal cycle. It is possible that the vagina may become infected in the process of taking smears and that the appearance of pus may make it impossible to interpret the smear. For this reason the use of the sterile pipette was preferred to that of the loop, which was considered to be more likely to traumatize the vagina. While the possibility that infection occasionally occurs in spite of this precaution cannot be ruled out, in our experience it rarely leads to difficulty in reading the smears of the normal cycle. It would therefore appear to be unlikely that it would invalidate a comparison between large groups of control and experimental animals smurred by identical techniques.

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EXPERIMENTAL

In a preliminary experiment an attempt was made to determine the minimal daily dosage required to maintain continuous vaginal oestrus. Thirty-three animals were injected daily for periods of several days with 1, 2, 3 and finally 4 units. Though the lower doses produced continuous vaginal cornification for short periods, 4 units by daily injection were required to maintain oestrus for the experimental period following such preliminary treatment. Having established this threshold, we injected 18 of these animals daily for 3 weeks with 300 units and maintained the remaining 15 of the group on 4 units daily over the same period of time. This was followed by a period of 26 days during which all animals of the group received 4 units daily. The smears of the control and experimental groups were compared over a period of 10 days between the 7th and 17th days following the drop in dosage of the experimental group from 300 to 4 units. This particular period of time was chosen in view of the fact that the response of the experimental group over the first week was obviously influenced by the previous high dosage. The sudden change in the appearance of the smear which occurred in 15 of the 18 experimental animals at the 7th day was taken as indicating the end of the prolongation of effect of the high doses. At the 17th day some of the control animals had begun to go out of oestrus, indicating that from this time onwards the threshold level of 4 units was no longer adequate. Each animal was placed in one of three groups according to the number of days of the 10 when the smear showed full cornification (see Table I).

Table I. *The response of ovariectomized mice to oestrogens after previous treatment with high doses*

Number of days of full oestrus in 10-day period	Number of animals in oestrus	
	Control group	Experimental group
0-2	2	13
3-5	2	3
6-10	11	2
Total	15	18

Of the 15 control animals, 11 were in full oestrus on 6 or more of the 10 days. Of the experimental group only 2 of 18 animals showed a similar response. These results indicate that following a course of 3 weeks' administration of high doses of oestrogen, the minimal dose required to maintain continuous oestrus is increased to a degree far greater than that of animals kept on threshold doses.

In order to investigate the extent to which the threshold level is modified by such previous treatment, a further 103 animals were prepared and divided into 5 groups as follows:

- (a) 32 animals injected for 3 weeks with 300 units daily followed by injections of 3 units daily for 26 days.
- (b) 17 animals injected for 3 weeks with 300 units daily followed by injections of 4 units daily for 26 days.
- (c) 12 animals injected for 3 weeks with 300 units daily followed by injections of 6 units daily for 26 days.
- (d) 10 animals injected for 3 weeks with 300 units daily followed by injections of 12 units daily for 26 days.
- (e) Control group. 32 animals injected over the 26 day period only with 3 units daily.

The results of these experiments are given in Table II.

Table II. *The extent of the change in the response of ovariectomized mice to oestrogens after previous treatment with high doses*

Number of days of full oestrus in 10-day period	Number of animals in oestrus				
	Control group (e)	Experimental groups (a) (b) (c) (d)			
0-2	1	13	13	8	2
3-5	3	11	4	3	4
6-10	28	8	0	1	4
Total	32	32	17	12	10

Injections of 3 units daily maintained continuous oestrus in 28 of 32 previously untreated animals (e) as compared with 8 of the 32 animals injected previously with 300 units daily for 3 weeks (a). The results obtained with the other groups, (b), (c) and (d), indicate that this change in threshold is considerable.

Table III represents a comparison between all the control and experimental animals of Tables I and II. Thirty-nine (83%) of 47 control animals were maintained in oestrus on 6 or more days of the experimental period as compared with 15 (16.9%) of 89 experimental animals on similar or higher doses.

Table III. *A comparison between the response to oestrogens of all the control and experimental animals referred to in Tables I and II*

DISCUSSION

The experiments show that the response of ovariectomized mice (as judged by the vaginal smear) to daily injections of oestrone is influenced by previous oestrogenic treatment. Similar evidence has also been obtained by Zuckerman [1941] in monkeys. Although Marriau & Parkes [1929] and Emmens [1939] concluded on the basis of their experiments that previous treatment did not affect the response of animals to single injections of oestrogens, Palmer (data to be published) working with mice has re-examined this problem and has found that the response to single injections is in fact related to previous response which in turn is determined by previous dosage.

These observations are in agreement with the fact that clinically we have noticed instances of decreased response following prolonged administration of oestrogens (e.g. in treatment of menopausal cases). This is a point of considerable clinical importance, since it indicates that large doses of oestrogens cannot be given indiscriminately without fear of increasing the threshold at which a minimal dose will produce the desired effect. The mechanism by which this decreased response occurs is not clear. D'Amour, Dumont & Gustavson [1934] found that serum taken from rats injected for periods of 4-8 weeks with 5-20 units of oestrogen exhibited no anti-oestrogenic properties. It would therefore appear that the effect is not the result of the production of an antihormone to oestrogen but may be due to a decreased response of the vaginal mucosa following prolonged treatment with high doses.

SUMMARY

(1) Minimal doses of oestrone which by daily injection produced continuous oestrus in ovariectomized mice did so for short periods only, and had to be followed by gradually increasing doses in order to maintain continuous cornification of the vagina.

(2) After a 3-week period of treatment with 300 units of oestrone by daily injection, ovariectomized mice showed a considerably decreased response to oestrone as compared with the response of control animals.

We take this opportunity of acknowledging our indebtedness to Mr. L. Westwood, our technical assistant, who has devoted considerable time, care and interest to this experiment.

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HENNY-FEATHERING IN BROWN LEGHORN MALES

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SOME years ago observations were published on a castrated Brown Leghorn male, one of a flock maintained at this Institute, which assumed female secondary sexual characters [Greenwood & Blyth, 1932]. This sex change involved both the head furnishings and the plumage, and a study of the relevant facts suggested that the phenomenon was referable to the presence of a small abdominal tumour of epithelial origin. Although reversal of external sex characters from female to male is by no means uncommon in fowls, only one case had been investigated previously, so far as could be determined, of a male bird showing development of female characters: this was a Silver Grey Dorking cock described by Buchanan [1926], who concluded that its transformation was related to a hypo-functioning thyroid. The recent appearance of plumage sex reversal in an otherwise normal male bird of our flock is the subject of the present contribution.

Although plumage sex dimorphism, dependent on endocrine action, is the commonest manifestation among the many varieties of domestic fowl as they exist to-day, there are a few strains in which this difference is not found and where both sexes have a type of feathering similar to that of the female of cock-feathered breeds. The Sebright bantam is an example of this type, while in the Hamburg and Campine breeds both cock-feathered and henny-feathered males occur.

From investigations as to the mode of inheritance of henny-feathering Punnett & Bailey [1921] concluded that it behaves as a dominant, differing from cock-feathering by a single mendelian factor. They criticized Morgan's [1919] interpretation of a two-factor difference on the grounds that his cross males were not kept long enough to obtain a real ratio, for many henny cocks pass through a phase of male feathering and only exhibit the female type after the first adult moult. (This, as will be shown later, was the course of events in the individual under discussion.)

In cock-feathered males, and in capons of henny breeds, both thyroid and sex hormones are capable of inducing structurally female plumage; in the Brown Leghorn male oestrogen can also induce the partridge pattern of the hen but thyroxine alone cannot do so. Whether the secretions of the ovary are the only ones which can effect this response is not clear, for it has recently been shown that implantation of relatively large amounts

of testis tissue also have this effect [Greenwood & Blyth, 1938b], but whether this is due to the quantity of male hormone present or to traces of female hormone associated with it is a matter for conjecture. Though capons of hen breeds respond to testosterone medication by feminization of the plumage, the only effects induced by large doses in Brown Leghorn capons were symptomatic of thyroid deficiency [Emmens & Parkes, 1940].

The many attempts to expose the relation between the physiological and genetical bases of hen-feathering have provided further information. Jull & Quinn [1930] were able to produce hen-feathered cocks of Brown Leghorn type by crossing Brown Leghorn hens with descendants of a hen-feathered male from Morgan's Sebright-Blackbreasted Game bantam cross. Skin and gonad cross transplantations between Sebrights and Brown Leghorns have revealed that hen feathering is a quality vested in the skin and not in the reproductive organs, and suggested that the difference lay in a lower threshold of plumage response in this type [Roxas, 1926; Greenwood, 1928; Danforth, 1928]. Callow & Parkes [1936] substantiated this by showing that much smaller quantities of oestrone are required to feminize Sebright capons than those of cock-feathered breeds.

CASE HISTORY

The bird in question, K1879, was hatched on 18 April 1938. It was derived from a highly inbred line (known as the S.E. line) in which the plumage of both sexes shows some characteristic deviations from that of the recognized Brown Leghorn type: in the female it is much paler than normal; the drab background of the dorsal contour feathers is of a lighter tone and the black pencillings more restricted. On the wing bow and edge of the secondaries the female pattern may be obliterated by a bay colour similar to that of the male secondaries. In regions normally pure black, however, such as the tail and inner web of the primaries, female pattern may occur. The males also have a lighter appearance, the melanization usual in the proximal part of many of the feathers on the neck and dorsal surface being much reduced and the black ventral feathers splashed with red to varying degrees. The red in the latter is of a deeper tone than the salmon of the female breast feather. In other regions, normally black in the male, such as sickles, posterior saddle and secondary coverts, varying amounts of female pattern are encountered. Even the secondaries may show the pattern, but here it involves the outermost part of the black area and not the bay edge. The general impression gained is that the amount of melanin in the plumage of this line is everywhere below normal, and is enhanced by the fact that the remaining black areas on these birds have a brownish tinge.

While in male plumage when penned with females in the spring of 1939, K1879 showed more extensive areas of female pattern than any of the males hitherto retained for breeding. The moult of the same year resulted in a marked expansion of the abnormality: by October all fringing had disappeared from the full-grown plumage except on the neck, and while the sickles were longer than their female homologues, they had blunt rounded ends and a very clearly marked female pattern; back and saddle feathers were typically light female; the breast was a mosaic of red and black, and black feathers, though the non-melanic coloration was deeper than is usual in hens. Only on the wing was the female pattern lacking: the wing bow and covert feathers were a dull red with varying amounts of stippling or pencilling at the tips. Since, however, blurring of the pattern, and reddening, often occur on the wing of normal hens, the entire plumage picture presented can be regarded as a close approximation to typical female, and had the bird belonged to a self-coloured breed the transformation must have been considered complete. New feathers were still growing in at this time, however, and these appeared to be differentiating in a male direction, fringing again being evident; breast feathers which had almost completed their growth were completely black. The return to its proper plumage type could be followed in December in areas that had previously been denuded of feathers, but the maleness was of the 'too red' variety peculiar to the strain.

This isolated occurrence of a hen-feathered male in a cock-feathered breed may not be so spontaneous as might at first appear, for it has been noted that in the highly inbred line in which it arose males frequently exhibited regions of female patterning. Up to the 6th and 7th generation back all his progenitors, except one, derived from the same male and female, and the exception, which appears once in the 4th, and once in the 5th generation, had one granddam which was a daughter of the same pair. The male of the original mating bore traces of female pattern, which characterizes so many of his male descendants, on his secondaries and posterior saddle feathers, and had a liberally red splashed breast. In two of K1879's immediate ancestors the abnormality was sufficiently evident to be noted: his paternal grandsire had stippling on the tail sickles and posterior saddle, and a slightly mis-marked breast, while the intervening sire showed femaleness on tail sickles, primaries, wing coverts and secondary tips, and had a mis-marked breast; the tail sickles had a reddish ground.

In the S.E. line fertility, hatchability, and rearability are considerably below the average for the flock, but apart from this family characteristic K1879 showed no abnormalities when used as a breeding sire. From his mating to ten closely related females 148 eggs were incubated between May

and October: of these 82% were fertile and 54% hatched (32 ♂♂ : 36 ♀♀). Ten of the males were retained for further observation; none of them exhibit so much femaleness in their plumage as their sire, but three, one from his dam and two from her full sister, show a very marked degree of melanin deficiency. A second cockerel bred from his dam shows the deficiency to about the same extent as his remaining offspring from slightly less related hens.

These cockerels are now on the verge of their first adult moult, and in all but one the new feathers show no extension of the deviation from the normal sexual type. The exception is further advanced in the process than the others and has developed a group of new posterior saddle feathers, female in structure and with a very clear female pattern as opposed to their red, fringed, and indefinitely stippled predecessors. Elsewhere on the body, growing feathers appear to be male in type.

EXPERIMENTAL

Though the case history suggests that the plumage abnormalities exhibited by K1879 are genetic in origin, there is also the possibility that they arose from physiological disturbances, and a preliminary examination has been made of this side of the question. All previous work has indicated that the thyroid gland is unable to induce the female pattern of the Brown Leghorn without the intervention of ovarian secretions; nevertheless, a theory (advanced in the discussion) developed to accommodate the known facts involved the supposition that abnormal relations between thyroid and plumage existed in this line. Accordingly, during 3 months from the beginning of March, dried thyroid powder (B.D.H.) was fed to the following birds:

K1879,

L2791, one of the less red of K1879's sons, showing femaleness on the tail coverts and a red splashed breast.

L2598, son of another S.E. male mated to a female of another line; very like L2791 except that he shows no female pattern.

L3291, a totally unrelated typical dark plumaged cockerel.

J1260, an unrelated thyroidectomized cock.

J3408, an unrelated thyroidectomized hen.

The thyroidectomized birds received a constant daily dose of 0.2 g. during the whole period; this was sufficient to support the regeneration of feathers of normal sexual type in place of the red fringed ones removed from dorsal and ventral regions. L3291, the normal cockerel, received 0.3 g. (daily) for 4 weeks, then 0.5 g. for 6 weeks, and finally 0.7 g. for 2 weeks. Hyperthyroidic effects followed the expected course: the barbed areas of feather vanes extended until the fringed areas were engulfed; this

occurred with lower doses in the cape and wing regions but was not completed in the saddle until the highest dose was administered. Concurrently there was an extension of melanization towards the feather tip until completely black solid-vaned feathers were obtained. In the cape feathers grown at lower dosages the tips were not completely black and the melanin was distributed in broken patches or blobs which showed no obvious relation to the pencilled pattern of the female; the ground colour was the red usual in the male cape.

K1879 received 0.3 g. for 3 weeks, then 0.5 g. for 6 weeks. Feather pluckings made throughout the winter showed that his plumage had remained typically male in structure, 'too red' dorsally and mixed black and red ventrally. Feathers removed during the experiment were replaced by typical female feathers on the back and wing, and black feathers on the breast. With the higher dosage black areas appeared on the tail coverts. Cessation of treatment was followed by a return to S.E. plumage type.

The two remaining birds, K1879's son and the crossbred, were both treated with a series of graded dosages: 0.3 g., 10 days; 0.2 g., one week; 0.3 g., 2 weeks; 0.5 g., 7 weeks; 0.7 g., 2½ weeks. The effect of hyperthyroidism in those two birds showed striking differences from those displayed by other treated individuals. The development of barbules extended right to the tip, even in saddle feathers, with the lower dosages, but while there was an increased deposition of melanin it was slower in appearing than in the normal male, so that among the first regenerated back and saddle feathers many had a solid vane, mainly red or yellow, with only a little broken black at the tip or along the side of the rachis. The red pigment had a paler bleached appearance compared with that in feathers grown before the treatment (perhaps due to a very slight deposition of melanin). In the saddle feathers the black was often nearly all on one side of the vane. The darker feathers of the cape, saddle and wing bow were also peculiar for in many the extreme tips were reminiscent of homologous female feathers: the melanin was arranged in fine stippling that occasionally merged into fine lines across the feather, and the ground colour was not red or yellow but the drab straw colour typical of the female. The patterned area was never more than a centimetre long, usually less, and in the majority of feathers the apparent femaleness was contradicted by splashes of red in the more proximal parts of the vane. A very few, however, had a solid black vane and it was found impossible to distinguish between these and similarly marked feathers removed from normal hens. Attempts to obtain an extension of the effect, or a more definite female pattern by increased dose were without success; one or two feathers from both birds, grown under the highest dosage, show patches of drab female ground colour in the centre part of the vane but they are as small as 1

irregular that to identify the black stippling in them as female pattern would be unjustified. The tail coverts and breast feathers of both birds grew in a solid black under the treatment.

DISCUSSION

First consideration of the plumage phenomenon displayed by K1879 suggested that it had a genetic basis, and that inbreeding or mutation had brought about an extension of the hereditary trait. By developing female feathering at the first adult moult K1879 resembles some of Punnett & Bailey's [1921] intermediate crosses, and its reversion to male is not without parallel in the same group. The colour and pattern aberrations in the female phase are also reminiscent of those described in their extracted Brown Leghorns. If, however, the character has been exposed by inbreeding it must be recessive to the normal type, while in the crosses just mentioned hen feathering behaved as a dominant. On the other hand, were it a dominant mutation, the interesting supposition emerges that it is a link in a chain of progressive mutations towards a hen-feathered type, for it is difficult to believe that it can be unconnected with the family tendency to femaleness in the plumage of the male. While the records of his progeny have produced no repetition of the phenomenon so far, the possibility of its hereditary nature cannot yet be set aside. Actually, while it was possible to reproduce it by thyroid treatment in K1879 himself, the fact that in his son the plumage reaction to this endocrine was different, not only from his sire, but from normal Brown Leghorns, adds to the evidence in favour of a genetical interpretation.

In the strain to which K1879 belongs it was noted that the traces of femaleness present in the males were always restricted to normally melanic feathers, and this led to the hypothesis that the males in question carried female pattern but were unable to express it over the whole body because of the unsuitable nature of the feathers. Or, to put it otherwise, the plumage picture could be referred to the action of a gynaecogenic agent in conjunction with a male level of thyroid activity: the fact that the breast feathers are never completely red suggests that the former is less effective than in the normal hen.

We have now evidence of three agents capable of inducing the Brown Leghorn female pattern under suitable conditions. In K1879, thyroid; in normal hens, oestrone (presumably associated with an adequate level of thyroid activity). The action of the third agent—testis—is still difficult to understand, since, though the present writers were able to induce female plumage by implantations of relatively large amounts of this gland, Emmens & Parkes [1940] could not do so with heavy doses of its active secretion (testosterone). Punnett & Bailey [1921], however, mention one of

their extracted Brown Leghorn henny males which was castrated and developed male plumage, indicating that in this bird testis was supporting the exhibition of female plumage. There is little doubt that thyroid is involved in all three cases, but the differences between them give cause to question whether sex hormones are absolutely essential for the expression of the female pattern. Our earlier work suggested that oestrone acted by restricting the increased melanin present [Greenwood & Blyth, 1938a]; this implies either a rhythmic action to produce the fine pencillings, or else that the pattern lines are potentially present in all Brown Leghorns of both sexes, but only show up in the presence of melanin restricted to a certain level of intensity, i.e., 'diluted'. On the latter theory then any condition which caused a low grade of melanin deposition over the whole feather would be expected to expose the female pattern. If, for instance, crossing Brown Leghorns with recessive white birds results in males with blue in place of black regions, the pattern might be expected to appear. It is hoped to test this later.

In the normal course of events thyroid administration is not productive of dilute melanization at any level of treatment; the effect is rather that of an extension of melanin deposition at a uniform high level of intensity further and further up the feather vane with progressive increases in dosage. The general lightness of the plumage in the S.E. birds suggested that either they were hypothyroidic, or more probably, since they are functionally fairly normal, that the plumage was exhibiting a refractoriness to the influence of this gland. The results of the preliminary thyroid feeding tests appear to favour the latter contingency, for neither K1879, his son, nor the cross bred showed any tendency to approach normal male colour distribution under the treatment; in the last two birds there was ample evidence that the increased deposition of melanin was lagging unusually far behind the structural changes, though they appeared more readily than in the normal cockerel. Our expectation that this poor reaction to thyroid might result in a dilute melanization, and so feminization of the plumage, has not been achieved, but neither has it been entirely refuted. It is still possible that, with a more finely graded series of dosages, some of the feathers with a bleached red appearance might have developed female pattern at one particular stage in the treatment, but since they always had some black markings the resulting appearance would obviously not be entirely female.

The fact that a condition closely approaching femaleness was obtainable in the tips of feathers of L2791 and L2598, however, lends support to the view that their plumage type stands in a position intermediate between that of K1879 and normal Brown Leghorn males, and that their peculiarities are of the same nature, but of a less extreme form than those of the former bird.

There is now the question of reconciling this conclusion of a hereditary basis for the phenomenon with the fact that in K1879 the plumage sex reversal was of a passing nature. Thyroid gland weight in fowls undergoes a rise and fall throughout the year, which may be taken as indicative of a reciprocal rhythmic cycle of functional activity. Statistical data from our own flock show that the lowest weights occur in the May-June period, i.e. just prior to the onset of the moult. Thus it may be that the highest point of thyroid activity in K1879 was just sufficient to induce the reversal and that with its later falling off the feathers regenerated according to their normal type. Though no doubt minor variations occur in the extent of the annual rhythm from year to year, it would be expected on this argument that some indications of a similar reversal would appear in subsequent moults. In K1879 the moult of the present year is commencing, and while some new back feathers again show female pattern all exhibit a reduction or absence of fringing.

If, however, the level of thyroid activity in this bird is within the limits of normality throughout the year, then to assume, as we have done above, that the melanin reaction to thyroid is poor is not entirely correct. The initial threshold of response of the feathers may be higher than in dark plumaged lines, but if our view of the mechanism of pattern formation is right, then it is in the distribution of small amounts of melanin that the critical differences lies. Small increments deposited evenly over the whole feather vane, instead of amassing proximally as is usual, would result in the dilute melanization which we have postulated is necessary for female pattern. Since this infers an optimum—not a maximum—level of thyroid functioning, further hyperthyroidism should act differently from the effect in the normal hen by obliterating the pattern again—a condition fulfilled in L2791 and L2598, and less definitely in K1879.

In the absence of any experimental tests no mention has been made of the possible role of testis in this case of plumage reversal. On analogy with findings in henny-feathered breeds its action may be indirect through the thyroid.

While the conclusions reached can only be tentative, the data is sufficient to suggest that in the S.E. line we have an interesting link between cock-feathered and hen-feathered strains, and one in which more detailed investigation might go far to elucidate the mechanism underlying the differences and relationships between the two types.

SUMMARY

During its first adult moult a cock, belonging to an inbred line of Brown Leghorns, developed female plumage; later in the year it reverted to male feathering. The birds of this line are peculiar in that the plumage colouring

is much paler than normal, and the males have a tendency to exhibit female pattern on the black feathers of their upper surface.

It was found possible to renew the plumage reversal in this bird by thyroid feeding. Similar treatment of related males revealed that their reaction to hyperthyroidism was also different from that of the normal; barbule extension was rapid but increased melanin deposition slow to appear. In dark feathers a pattern resembling the female type was exhibited at the tip.

The data available suggest that the phenomenon is of a hereditary nature, based on a variation in the kind of reaction to thyroid hormone.

A theory is put forward that the expression of Brown Leghorn female pattern in either sex is not necessarily always dependent on the direct action of sex hormones, but will occur under any conditions which provide a certain low level of melanin distribution in the feather vane.

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THE EFFECT OF TESTOSTERONE ON THE RESPONSIVENESS OF THE IMMATURE GONAD TO CHORIONIC GONADOTROPIN

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IN a previous communication [Selye, 1940] we described the severe atrophy which is produced by testosterone in the ovaries of immature rats. It has also been shown that in rats younger than eighteen days gonadotropic extracts of pregnancy urine cause only theca luteinization but no follicle maturation or corpus luteum formation. It was found, furthermore, that the maturation of follicles up to the stage of antrum formation appears to be independent of the hypophysis since it is not interfered with by hypophysectomy. Hence it was concluded that the luteinizing hormone of pregnancy urine (chorionic gonadotropin) is able to stimulate the follicles only after the ovary has reached a certain stage of maturation [Selye & Collip, 1933; Selye, Collip & Thomson, 1933, 1935]. The fact that the atrophic ovary of the hypophysectomized rat likewise responds only with theca luteinization and reveals no follicle maturation or corpus luteum formation after treatment with such pregnancy urine preparations substantiated this conception [Noguchi, 1931; Collip, Selye & Thomson, 1933*a, b*; Collip, Thomson & Selye, 1933].

It appears probable that in order to obtain corpus luteum formation with chorionic gonadotropin a pituitary principle not present in pregnancy urine (probably the follicle stimulating factor—FSH) must sensitize the ovary. In the case of prepubertal rats this pituitary factor is furnished by the animal's own hypophysis, while in animals younger than 18 days the hypophysis is apparently so immature that chorionic gonadotropin cannot stimulate it to produce FSH [Selye & Collip, 1933].

In view of these considerations, it appeared of interest to establish whether the atrophic ovary of the testosterone-treated immature rat which contains only a few immature follicles would respond to chorionic gonadotropin with follicle maturation and corpus luteum formation or whether—like that of the hypophysectomized rat—it would only react with theca luteinization. By establishing this we hoped to decide whether it is the condition of the ovary or that of the hypophysis which determines the action of chorionic gonadotropin. The ovary of an immature rat receiving prolonged treatment with testosterone is quite comparable to that of a hypophysectomized animal inasmuch as the follicles reach only

very early stages of maturation and then undergo atresia while their theca cells are transformed into the so-called 'wheel-cells' which are characteristic of hypophyseal deficiency. If the response of the ovary is conditioned by the degree of its development, the gonad of testosterone-treated animals should only respond with theca luteinization just as that of hypophysectomized animals. If, on the other hand, its reactivity is dependent upon the production of a synergistic pituitary principle, it should be able to form mature follicles and corpora lutea after chorionic gonadotropin treatment, assuming of course that testosterone does not interfere with the normal responsiveness of the animal's own pituitary.

EXPERIMENTAL

In order to answer these questions a group of female albino rats was treated daily with 1.0 mg. of testosterone propionate in 0.05 ml. of peanut oil intraperitoneally for 14 days beginning on the second day of life. After that, treatment with the same dose was continued for 16 more days but the injections were administered subcutaneously. It was necessary to administer the hormone intraperitoneally during the first two weeks of life because the soft skin of very immature rats is not elastic enough to prevent regurgitation of the oily solution through the needle punch. Another group of eight females of the same age was similarly treated with peanut oil only. On the 30th day of treatment one ovary was removed from three animals of each group by laparotomy for histological examination, and after this each of the sixteen rats was given six daily subcutaneous injections of 100 I.U. of 'Physex', a chorionic gonadotropin preparation which contained 15,000 I.U. per gramme. At the same time, the daily oil and testosterone propionate injections were continued in the two groups in the same manner as prior to the initiation of the gonadotropin treatment. All animals were killed on the 7th day so that each of the gonadotropin-treated rats received a total of 600 I.U.

Histological examination of the ovaries removed by biopsy—that is before the chorionic gonadotropin treatment was started—showed that in the peanut oil-treated controls (Plate I, Fig. 1) the average ovarian weight was 15 mg. (range: 13.6–19.1 mg.), and although no corpora lutea were detectable in these prepubertal animals, the follicles were well developed, several of them showing fairly large antra. The theca cells were of normal appearance. In contradistinction to these the ovaries of the testosterone-injected rats (Plate I, Fig. 2) had an average weight of 6 mg. (range: 4.5–7.1 mg.) and revealed only a few small follicles, most of which were atretic. The theca cells were transformed into 'wheel-cells' in most of the follicles whose granulosa underwent atresia. The ovaries show a striking resemblance with those of hypophysectomized rats except that in the

hilum region they contain the typical medullary cords which have previously been described in testosterone-treated immature rats [Selye, 1940] and are considered to be a sign of partial masculinization of the gonad. After the gonadotropin treatment the ovaries of the control animals (that is, those pretreated with peanut oil only) were greatly enlarged, their average weight being 90 mg. (range: 68–110 mg.) and showed numerous recent corpora lutea (Plate I, Fig. 3). The ovaries of the testosterone-pretreated group, on the other hand, were less conspicuously enlarged, having an average weight of 18 mg. (range: 12–27 mg.), but upon histological examination all of them showed recent corpora lutea and in most cases several 'blood points', that is, haemorrhages into the central cavities of newly formed corpora lutea (Plate I, Fig. 4).

From these experiments we may conclude that even though the very atrophic ovary of testosterone-treated immature rats does not enlarge as much under the influence of chorionic gonadotropin as the ovary of a normal animal, it responds essentially in the same manner, that is to say, with follicle maturation and luteinization. It is evident that the ovary of a testosterone-treated animal cannot respond quantitatively as well as that of a normal control since it contains fewer follicles capable of luteinization. Yet the fact that the formation of true corpora lutea is still possible in these ovaries under the influence of chorionic gonadotropin—which causes only theca luteinization in the similarly involuted gonads of hypophysectomized rats—proves conclusively that the inability of luteinizing hormone to transform the granulosa of the hypophysectomized rat into a corpus luteum is not due to the atrophic condition of the granulosa itself but to the absence of the pituitary. This confirms our theory [Selye *et al.*, 1933, 1935] according to which the normal action of chorionic gonadotropin is due to its ability to stimulate the animal's own hypophysis to produce another gonadotropic hormone, which acts synergistically with the pregnancy urine principle. The above experiments show, furthermore, that although testosterone inhibits the gonadotropic hormone production of the hypophysis, it does not prevent this gland from producing this synergistic gonadotropic principle when chorionic gonadotropin is administered, and does not render the gonad unresponsive to gonadotropic hormones.

In a second group of sixteen one-day-old male albino rats we repeated this experiment in exactly the same manner as in the above females, hence a detailed description of the experimental conditions is unnecessary. We wish to mention, however, that under the influence of testosterone the average testis-weight decreased from the normal of 892 mg. (range: 700–1330 mg.) to 348 mg. (range: 250–500 mg.) and that both the interstitial cells and the seminal epithelium underwent severe atrophy. However, spermiogenesis was not completely inhibited in all cases. After the

gonadotropin treatment the average testis-weight of the controls showed only a slight increase to 922 mg. (range: 870-1525 mg.), while that of the testosterone-pretreated males rose more markedly to an average of 761 mg. (range: 520-839 mg.). The seminal epithelium showed only slight stimulation, while the interstitial cells were greatly enlarged under the influence of chorionic gonadotropin, both in the testosterone-pretreated and in the control animals.

It is of some interest to note that testosterone does not prevent the gonadotropic action of chorionic gonadotropin in males, but this observation is far less important than the corresponding finding in females, since even hypophysectomy does not abolish the responsiveness of the male gonad to such preparations.

SUMMARY

Experiments in immature rats indicate that, although testosterone propionate causes an ovarian atrophy comparable to that produced by hypophysectomy, it does not interfere with the normal responsiveness of the ovarian follicles to chorionic gonadotropin (gonadotropic pregnancy urine preparations). Since hypophysectomy prevents the response of the follicles under similar conditions, it is concluded that the irresponsiveness induced by ablation of the pituitary is not due to the atrophy of the gonad. It appears more likely that chorionic gonadotropin is dependent for its action on the follicles upon the compensatory secretion of another gonadotropic principle (probably the follicle-stimulating factor) by the animal's own pituitary.

The experiments herein reported show furthermore that testosterone does not induce ovarian atrophy by direct action on the gonad itself but by inhibiting the normal secretion of hypophyseal gonadotropic hormones. Chorionic gonadotropin can stimulate the secretion of gonadotropic pituitary hormones even in animals in which the normal secretion of such hormones is inhibited by testosterone.

In immature male rats, in which the interstitial cells underwent atrophy as a result of testosterone propionate treatment, chorionic gonadotropin may not only restore these cells to normal but cause them to undergo hypertrophy. The seminal epithelium, on the other hand, shows less pronounced atrophy under the influence of testosterone and is less readily stimulated by chorionic gonadotropin.

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FIG. 1. Ovary of untreated immature control rat. $\times 48$.



FIG. 2. Ovary of testosterone-treated immature rat. Note the large number of degenerating follicles whose theca is atrophied. The few fairly large follicles are in the process of atresia. Medullary cords are seen along the hilum on the right side of the photograph. $\times 48$.



ANTIGONADOTROPHIN IN THE SERUM AND MILK OF THE FEMALE GOAT

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IN animals receiving gonadotrophic hormone regularly for a comparatively long time, the appropriate effect on the gonads may, under certain conditions, be induced during only a relatively short part of that time.

In such conditions an initial period of increased endocrine function of the gonads is followed by a period of inactivity and ultimately by atrophy [McPhail, 1933]. *Pari passu* with the development of this refractory state the blood serum acquires the capacity to inhibit the activity of the gonadotrophic hormone in question, if the serum and hormone are injected into animals at separate sites.

The conditions for development of antigonadotrophic sera require that the gonadotrophin shall have originated in the tissues of a species other than that of the animal yielding the serum.

We found these antisera useful in identifying the species of origin of unknown gonadotrophic preparations, for example to see whether they had been extracted from pituitaries or from human pregnancy urine [cf. Selye, Baelman Thomson & Collip, 1934; Selye, Collip & Thomson, 1934 *a, b*]. So as to have a continuous supply of fresh antiserum we treated 3 goats with pregnancy urine extract for more than 2½ years, during which time the serum concentration of antihormone was checked repeatedly. We also made a number of observations on the sexual activity of animals so used.

MATERIAL AND METHODS

Three adult female goats were injected every alternate day between 31 May 1937 and December 1939, with approximately 1,000 I.U. of chorionic gonadotrophin ('Pregnyl', Organon).

The antigonadotrophic goat serum was tested on immature female rats, 20 I.U. of chorionic gonadotrophin being injected in the course of 3 days into the left flank and the serum into the right flank. A test was regarded as positive if none of the six animals used for each dose of serum showed any ovarian change, enlargement of the uterus, or cornification of the vagina. Our unit of serum activity was the minimal amount necessary completely to inhibit the activity of 20 I.U. of the gonadotrophin.

RESULTS

Antigonadotrophin content of the serum

Pooled samples of serum of the 3 test goats were examined repeatedly with the result given in Table I.

Table I. *Amount of antigonadotrophic goat serum necessary to inhibit the response of immature female rats to the injection of 20 I.U. chorionic gonadotrophin*

Duration of treatment (days)	Dose of serum required to neutralize 20 I.U. chorionic gonadotrophin (ml.)
47	0.19
86	0.075
123	0.04
172	0.08
231	0.05
299	0.06
353	0.04
556	0.06
592	0.07
653	0.07
918	0.05

After approximately 100 days' treatment the sera reached a maximal antigonadotrophic value, which further treatment did not increase.

Sexual behaviour of the goats

No signs of oestrus appeared in any of the goats during the first year of treatment. In the second year all three came into oestrus. They were mated on about the 525th day, at which point 0.06 ml. of serum inhibited 20 I.U., i.e. contained one unit of antigonadotrophic activity.

Pregnancy began at once in 2 of the goats, but the third was mated at several oestrus periods without conception. The antigonadotrophic activity of the serum remained throughout pregnancy at the same level as before mating.

The offspring were: 1 stillborn fully-developed kid delivered on 21 March; and 2 kids delivered on 3 June, of which one was fully developed but the other had died some time before. All of them were extremely emaciated. It is possible that the withdrawal of 150 ml. of blood from each animal shortly before delivery had a bad influence on the developing foetus.

The serum of one of the kids was collected 12 hours after its death and 0.4 ml. were injected with 20 I.U. of chorionic gonadotrophin into immature female rats. No inhibition was found: all the rats had a cornified vaginal smear and all their ovaries were luteinized. It is therefore probable that the antigonadotrophin in the maternal serum does not pass through the placenta.

The gonadotrophin given to the mother had not produced any traceable effect on the ovaries or uterus of those kids which were examined histologically.

Antigonadotrophin in milk

Lactation set in normally in both mothers. The antigonadotrophic concentration of the serum was unaffected by either delivery or the onset of lactation.

The milk was tested on immature female rats by the same method as the serum, our control being the milk of a goat that had had no gonadotrophic therapy of any kind. The results obtained are given in Table II.

Table II. *Effect of simultaneous injection of milk from goats injected with chorionic gonadotrophin on the response of immature female rats to the injection of 20 I.U. of the same gonadotrophin*

	Dose of milk (ml.)	No. of rats	% rats in oestrus	% of luteinized ovaries	Mean wt. of uterus (mg.)	Mean wt. of ovaries (mg.)
<i>Control</i>	1.2	12	64	100	94	20
	3.6	6	73	100	72	25
<i>Experimental</i>	0.3	12	78	100	82	19
	0.6	12	84	50	88	15
	1.2	36	31	24	47	10
	1.8	18	23	0	22	9

Each rat received 20 I.U. of chorionic gonadotrophin, and it is clear that the activity of this amount was totally inhibited by about 2 ml. of milk from the treated goats, whereas the control milk did not inhibit the reaction at all.

Since the daily milk yield was approximately 1,000 ml., which is capable of inhibiting the activity of 10,000 I.U. of the gonadotrophin; and since the goats had received only 1,000 I.U. on alternate days: it is also clear that these animals had produced much more antihormone than was needed to counteract the foreign gonadotrophin given.

By the 300th day post partum, when the milk yield had fallen to 200 ml., 2.0 ml. still inhibited 20 I.U. of chorionic gonadotrophin.

Absence of antigonadotrophin from urine

Before we found out how relatively high was the antihormone excretion in the milk, we tried to demonstrate its presence in the urine. Fresh urine being quite inactive under our conditions of assay, we made a concentrate by adding ethanol and dissolving in water the precipitate so obtained. But this concentrate was toxic, so that all the experimental animals at high dosage levels died. We therefore reduced the dose of gonadotrophin

hormone given with the serum to the rats and by so doing were able to investigate combinations of 33 ml. urine with 20, 10 or 5 I.U. of the gonadotrophin. In no case was any inhibition of gonadotrophic activity demonstrable.

The precipitate used in these experiments was treated with barium hydroxide and thereby so purified that we could test the equivalent activity of 200 ml. of urine. This was injected simultaneously with 10 and with 15 I.U. of the gonadotrophin, without success.

We also tried to investigate an ammonium sulphate precipitation of urine, but failed because all animals died during the treatment.

DISCUSSION

Several authors have noticed that if gonadotrophic hormones from another species be injected for a long time, ovarian activity is at first increased (multiple corpus luteum formation, uterine and vaginal changes) and later inhibited (exhaustion atrophy). Factors appear in the blood which can prevent even the initial activity of these gonadotrophic hormones in fresh animals. Thompson [1939] recently stated that the serum of a dog, immunized over a period of $3\frac{1}{2}$ years to an extract of sheep pituitaries, induced abortion in bitches injected in the last stage of pregnancy. In contradiction, Zondek, Hochman & Sulman [1939], found that the serum of a goat immunized over a period of 9 months to a mixture of pregnancy urine, anterior pituitary extracts and retroplacental blood (all of human origin), failed to prevent oestrus in female rats; during the time of administration of antiserum these animals were mated, pregnancy was normal and no abortions occurred. The antiserum, however, proved to be highly active in immature female rats.

The three goats described in this paper received a gonadotrophic preparation of human pregnancy urine over more than 2 years. During the autumn of the first year they showed no sign of sexual activity; the ovaries were probably atrophic as in other animals treated for several months. The serum then had a high value for the antigonadotrophic factor.

During the second year the sexual behaviour again became indistinguishable from that of untreated goats, all were mated, and two of the three became pregnant. The antigonadotrophic serum value remained uniformly high up to the end of the experiment; clearly therefore the high concentration of antigonadotrophic activity against a 'foreign' (in this case human) gonadotrophin had no adverse effect on the progress of oestrus or pregnancy.

In all probability the antigonadotrophic factor does not pass through the placenta, since none was found in the blood of one of the newborn kids.

Passage through the kidneys cannot with finality be excluded, since

although we failed to demonstrate its presence in the urine, we had been forced to work with urinary concentrates and conceivably the factor was destroyed by our method of extraction.

It must pass somewhat freely into the milk, since 1 litre of milk was sufficient to inhibit the activity of 10,000 I.U. of chorionic gonadotrophin. Milk is therefore a good starting material for the extraction of the antigonadotrophic factor.

The serum values reach a constant level after about 100 days' treatment by our method (constant dosage of gonadotrophin throughout the whole experiment). But since the antigonadotrophic factor is daily excreted in large quantity during lactation, it is clear that the formation of this factor must go on continually, at least in the lactating animal.

We are now trying to see how long this production goes on after gonadotrophic treatment is at an end.

SUMMARY

1. Three goats were treated for more than 18 months with 1,000 I.U. every other day of a gonadotrophic extract from human pregnancy urine.

2. During the autumn of the 1st year sexual activity was in abeyance, but in the 2nd year all the animals came on heat and mated. Two were pregnant until term, but the kids were born dead.

3. The antigonadotrophic activity of the serum of these goats was repeatedly tested on immature female rats. The values rose during the first 100 days and then remained more or less constant at a point where about 0.6 ml. thus inactivated 20 I.U. of the chorionic gonadotrophin.

4. The blood of the young showed no antigonadotrophic activity.

5. The urine of the adults showed no antigonadotrophic activity.

6. The milk of these animals was strongly antigonadotrophic, 1 litre of milk totally inhibiting the action of 10,000 I.U. of chorionic gonadotrophin in the immature female rat.

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THE CURVE OF ELIMINATION AND EXCRETION OF CHORIONIC GONADOTROPHIN DERIVED FROM THE RATE OF HORMONE RECOVERY AND ANTIHORMONE CONSUMPTION

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It is well known that the gonadotrophic hormone from pregnancy urine (chorionic gonadotrophin) rapidly disappears from the blood after its injection. Lipschütz & Vivaldi [1934] recovered only 20% 6–8 hours after its intravenous injection in the rabbit, and only 10% after 10 hours. Stamler [1937] who conducted similar experiments on dogs found that 3 hours after intravenous injection, only 38·4% remained in the blood. The hormone appeared in the urine within 1 minute of its administration, its excretion continued for over 20 hours, the total amount excreted in the urine being 11·2%. In the gelding, Stamler could find in the urine only 5% of administered chorionic gonadotrophin.

The investigations of Zondek [1935] have shown that up to 5% of subcutaneously injected chorionic gonadotrophin may be excreted in the urine in the rabbit or rat; and in human beings such excretion may reach 10%.

EXPERIMENTAL

Urinary excretion of chorionic gonadotrophin

We have recently checked these results on rats, employing another technique: 5 female rats had their urinary bladder ligated between the collum vesicae and the vagina. Immediately afterwards 1000 I.U. of chorionic gonadotrophin ('Korotrim') were given subcutaneously in 0·5 ml. normal saline. At intervals of 1, 2, 4, 20 and 24 hours the rats were killed and the urine collected from their bladders and assayed for chorionic gonadotrophin. After 4 hours we found 1%, after 20 hours 2%, of the amount administered. In a second assay on 5 male rats, we ligated the urethra subcutaneously and immediately afterwards the rats were injected with the same amount of gonadotrophin dissolved in 2 ml. of distilled water in order to cause a strong diuresis. At intervals of 1, 2, 4, 20 and 24 hours the rats were killed, their bladders emptied and the chorionic gonadotrophin in the urine determined. After 20 hours we found 5% of the injected gonadotrophin; before and after that interval none was found.

Destruction of chorionic gonadotrophin in the body

Having thus established that chorionic gonadotrophin is excreted in the urine only to a small extent, we proceeded to investigate the amount of hormone detectable in the body at various intervals after injection.

In 1934 one of us [Zondek, 1934 *a, b*] devised a simple method of investigating the rate of decomposition of oestrogenic hormones in the organism. This method has been extended to progesterone [Zondek, 1939] and to chorionic gonadotrophin [Zondek, 1940]. It consists of subcutaneous injection of immature animals with the hormone and the subsequent extraction of the whole animal (Method I). Another method was also proposed [Zondek, 1940] in which a certain amount of chorionic gonadotrophin is injected in immature female rats and after a certain interval smaller amounts of an antiserum to the gonadotrophin are given. The minimal dose of antihormone required to prevent the gonadotrophic reaction at any interval indicates the amount of chorionic gonadotrophin circulating in the body at that time (Method II).

Technique. Method I

Immature male rats weighing about 30 g. received one subcutaneous injection of 250 I.U. of chorionic gonadotrophin. Then they were killed at intervals of 0, 1, 4, 8, 12, 18, 24 and 30 hours. The bodies were cut into pieces and minced. Five times the amount of acetone was added to the resultant mash and well stirred, the acetone being 3 times poured off and fresh added. The residue was ground until a fine dry powder resulted. This powder was extracted with 75 ml. N/20 NaOH for 4 hours, with 75 ml. of N/20 HCl for another 4 hours and finally with 50 ml. of distilled water for 1 hour. These 3 extracts were then mixed, neutralized, washed with ether, centrifuged, made up to 200 ml. and tested on infantile female rats for their content of gonadotrophic hormone [cf. Zondek, 1940].

Technique. Method II

Antiserum which is given subcutaneously in immature female rats within 24 hours after the administration of chorionic gonadotrophin is able to check the gonadotrophic reaction [Zondek & Sulman, 1937 *b*]. It has been demonstrated [Zondek & Sulman, 1937 *c*] that chorionic gonadotrophin-antiserum neutralization follows strict quantitative laws which allow an exact standardization. With the aid of these methods it has been shown [Zondek, 1940] that 24 hours after injection of the gonadotrophin in the white rat only 10% may be recovered. On the basis of these results we have proceeded to draw a curve of chorionic

gonadotrophin decomposition in the body. 5 I.U. were injected in 7 groups of 5 immature female rats. After intervals of 0, 1, 4, 8, 12, 18, 24 and 30 hours the rats were injected with varying amounts of antiserum, which were able to neutralize 5, 3.75, 2.5, 1.25 and 0.5 I.U. of gonadotrophin respectively. The antiserum had been drawn from a goat which had been treated with subcutaneous injections of 2500 I.U. of chorionic gonadotrophin daily for a period of one year. The serum of this animal had an antigonadotrophic titre of 2000 anti-units per ml.,¹ it was preserved according to the method of Zondek & Sulman [1937 a] in the form of an acetone-dry powder, 70 mg. of which represented 2000 anti-units. With the aid of this preparation we proceeded to investigate the amount of antigonadotrophin required to obtain complete neutralization of the gonadotrophic reaction at various intervals after the injection of chorionic gonadotrophin. The amount of antihormone required allows of a direct estimation of the amount of gonadotrophin still circulating in the rat. One typical experiment is shown in Table I.

The synopsis of the results obtained with methods I and II tabulated in Table I demonstrates the rate of the decomposition of chorionic gonadotrophin within the body. The results obtained for the gonadotrophic reaction I (vaginal oestrus) were generally the most reliable. Rat 1 (method I) showed that the gonadotrophin injected into the assay rat could be recovered 100% by our extraction method. Rats 1-5 (method II) were set up to check the titre: they showed that if gonadotrophin and antigonadotrophin were given simultaneously, 5 I.U. of the former were neutralized by 5 anti-units of the latter. Rat 8 (method II) proves the existence of a maximum of 2.5 I.U. in the body 1 hour after the injection of 5 I.U. which corresponds to the 50% recovery in method I. Rat 14 of method II demonstrates that after 4 hours more than 1.25 anti-units are required to inactivate the previously injected 4 I.U. of gonadotrophin; this is in accordance with the 30% recovery in method I.

Similarly 8, 12 and 15 hours after the injection of 5 I.U. of gonadotrophin, more than 1.25, 0.5 and 0.5 anti-units respectively had to be given to inhibit the gonadotrophic reaction; these figures also agree with the 25%, 20% and 15% recoveries found by method I.

It was found that 24 hours after the chorionic gonadotrophin injection the antihormone was ineffective (Rats 31-35) owing to the fact that the gonadotrophic reaction of the ovaries had then started and the oestrogen produced by the matured follicles had entered the blood producing oestrus. The injection of even 40 anti-units at this time did not inhibit the \pm vaginal reaction. Thirty hours after the gonadotrophin injection

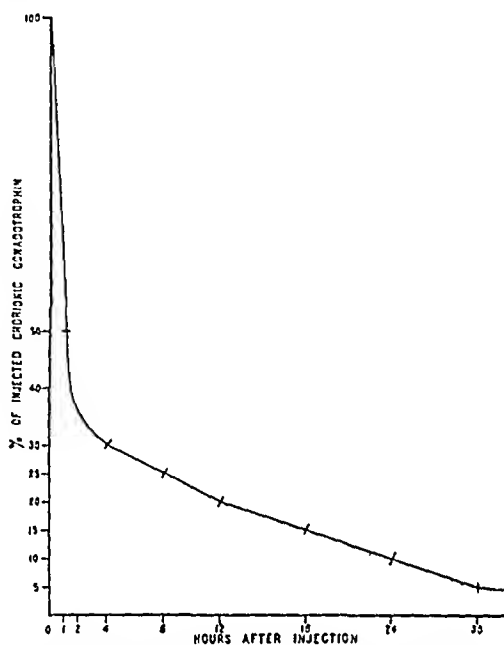
¹ 1 anti-unit is the amount necessary to neutralize the effect of 1 I.U. of chorionic gonadotrophin in the immature female rat.

the gonadotrophic reaction was more pronounced and method II quite inapplicable. Method I shows that at these times only 10% and 5% of the injected hormone were still present in the body.

Table I. *Amount of chorionic gonadotrophin (CG) circulating in the body at different intervals after injection*

Method I		Method II						
Assay rat no.	CG recovered by extraction	Assay rat no.	CG injected first (I.U.)	Anti-CG subse- quently injected (anti-units)	Interval between injections (hours)	Gonadotrophic reaction I Vaginal oestrus	II Blood points	III Corpora lutea
1	100% after 0 hour	1	5	5	0	—	—	—
		2	5	3.75	0	+	—	—
		3	5	2.50	0	+	—	+
		4	5	1.25	0	+	—	+
2	50% after 1 hour	5	5	0.50	0	+	+	+
		6	5	5	1	—	—	—
		7	5	3.75	1	—	—	—
		8	5	2.50	1	±	—	—
3	30% after 4 hours	9	5	1.25	1	+	—	—
		10	5	0.50	1	+	—	+
		11	5	5	4	—	—	—
		12	5	3.75	4	—	—	—
4	25% after 8 hours	13	5	2.50	4	—	—	—
		14	5	1.25	4	+	—	—
		15	5	0.50	4	+	—	—
		16	5	5	8	—	—	—
5	20% after 12 hours	17	5	3.75	8	—	—	—
		18	5	2.50	8	—	—	—
		19	5	1.25	8	±	—	—
		20	5	0.50	8	+	—	—
6	15% after 18 hours	21	5	5	12	—	—	—
		22	5	3.75	12	—	—	—
		23	5	2.50	12	—	—	—
		24	5	1.25	12	—	—	—
7	10% after 24 hours	25	5	0.50	12	+	—	—
		26	5	5	18	—	—	—
		27	5	3.75	18	—	—	—
		28	5	2.50	18	—	—	—
8	5% after 30 hours	29	5	1.25	18	—	—	—
		30	5	0.50	18	±	—	—
		31	5	5	24	±	—	—
		32	5	3.75	24	±	—	—
9	5% after 36 hours	33	5	2.50	24	±	—	—
		34	5	1.25	24	±	—	—
		35	5	0.50	24	±	—	—
		36	5	5	30	+	—	—
10	5% after 42 hours	37	5	3.75	30	+	—	—
		38	5	2.50	30	+	—	—
		39	5	1.25	30	+	—	—
		40	5	0.50	30	+	—	—

The initial rapid elimination and subsequent gradual decrease in the amount of hormone present in the body are graphically shown in the graph derived from the results obtained by method I.



Rate of elimination of injected chorionic gonadotrophin in the immature female rat.

DISCUSSION

It should be stressed that the very same curve, as drawn above, based on the rate of gonadotrophin elimination if small quantities (5 I.U.) are injected (method II), could be drawn from the rate of decomposition of chorionic gonadotrophin if large quantities are employed (250 I.U.—method I). It seems that there exists a general rule of inactivation: an excessive amount of hormone administered to the body is reduced within one hour to half of its original amount, and when the irreversible reaction of the stimulated organ starts after 24–27 hours, only a small part (10%) of the hormone remains in the body. The rest has meanwhile been eliminated without appearing in the urine. This phenomenon may be called: ‘Initial drop of the latency period’, which may be defined as the rapid reduction of the excess in hormone administered to the body, to a minimum level, before the gonadotrophic reaction itself sets in. This rule seems also to be true, with regard to sex hormones such as oestrone and progesterone [Zondek, 1934 *a*, *b*, 1939].

Regarding the fate of chorionic gonadotrophin in the body one problem is still to be clarified: if 50–70% disappears from the organism 1–4 hours after a subcutaneous injection, and if only 1–5% appear in the urine—

what has happened to the 49–65% left in the organism? In answer to this problem we may refer to the investigations of Gordon and his collaborators [Gordon, Kleinberg & Charipper, 1937 *a*, *b*, 1939; Gordon, 1937] who assume that decomposition of the gonadotrophic hormone is carried out in the reticulo-endothelial system.

SUMMARY

The curve of excretion of chorionic gonadotrophin in the urine following subcutaneous administration in rats, having their urinary bladder ligated, has been studied. The curve rises slowly and in the course of 4 hours only 1% of the hormone is excreted. The whole excretion during 20 hours amounts to 5% of the gonadotrophin introduced. Beyond this stage no excretion seems to occur.

The curve of elimination of the hormone from the body was quite different and has been studied by: I—the method of extraction of chorionic gonadotrophin from treated rats, and II—the method of subsequent administration of antihormone to rats previously treated with chorionic gonadotrophin. The most striking feature of this curve is the initial fall in the amount of the chorionic gonadotrophin injected, which brings about a decrease of 50% within the first hour. Later on the curve declines more gradually to reach a 10% level 24 hours following the subcutaneous injection. At this time the ovary commences to undergo irreversible gonadotrophic changes. The initial rapid decrease of the chorionic gonadotrophin in the body may be termed the ‘initial drop of the latency period’.

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THE INUNCTION OF SEX HORMONES ON THE SKIN

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MORE than a decade ago, Zondek [1929] showed that oestrone is absorbed by the skin. He applied the hormone in oil or ointment to the shaved skin of mice, and found that 7 times the amount which produced vaginal cornification by injection would do so by inunction. Later [1935 *a*] he reported that, following the inunction of oestrone in ointment to the skin of guinea-pigs, first the locally-treated mamma enlarges, followed by those more distant from the site of inunction. An oestrogenic ointment ('Oestraglandol') was also used with success in the local treatment of pruritis vulvae and acne vulgaris, and in the treatment of menopausal symptoms, in the human female [Zondek, 1935 *b*].

It seemed clear, therefore, that oestrone absorbed percutaneously is able to exert its normal effects throughout the body, but that it tends to act more effectively at the site of inunction if responsive tissue is there present, than elsewhere, at any rate when given in an oily or fatty base. This might have been predicted from such results as those of Fussgänger [1934], who first showed that the application of androgens directly to the comb of capons is a highly efficient method of stimulating comb growth, a small fraction of the amount required by injection being sufficient to produce a given increase in size. Jadassohn, Uehlinger & Margot [1938 *a*, *b*], and MacBryde [1939], also showed that, in women, the inunction of an oestrogenic ointment to one breast is followed by a greater response in the treated breast than in the other breast, receiving the ointment base only.

The investigations referred to above were carried out with oil or ointment as the medium carrying the oestrogen. An inquiry into the effectiveness of other solvents was made by Ito, Hajazu & Kon [1937], using vaginal oestrus, uterine and mammary responses of rats or rhesus monkeys as criteria. They found that the inunction of oestrone was most effective in 60% alcohol, the next best solvents being hydrous wool fat and then petrolatum.

Zondek [1938] extended these results and showed that the production of vaginal cornification in spayed female mice is as effectively realized by the inunction of oestrone in benzol, ether or 96% alcohol as by injection in oil, and that these solvents are far superior to oil for inunction. The

same superiority of the organic solvents was also seen when impairment of growth and antimasculine effects were used as criteria, the inunction of oestrone in these solvents proving as effective as injection in oil. In women, inunction of a tincture of oestrone or oestradiol benzoate in 96% alcohol was nearly as good as injection of the same quantity of oestrogen in oil.

Deanesly & Parkes [1937], using minimal doses, had meanwhile concluded that testosterone and testosterone acetate or propionate, when given by inunction in oil or propylene glycol, are not highly effective in restoring the prostate and seminal vesicles of castrated male rats. Moore, Lamar & Beck [1938] compared the actions of testosterone and its propionate when applied by inunction in a 'lanolin-like menstruum' or in cream to the skin of male rats and guinea-pigs. Using very large doses they concluded that the free hormone is superior by this route to the esterified form, and emphasized the ready absorption of both androgens and oestrogens through the skin.

Further work with male hormones, in the clinical field, has been carried out by Foss [1938, 1939]. He compared the effects of testosterone and testosterone propionate when administered by injection in oil, and superficial application in 'ointment' and in 96% alcohol, on a eunuch, a cunuchoid and a case of delayed puberty. He concluded that 2-3 times the injected dose of testosterone propionate must be applied as an ointment, and about 6 times as a tincture in alcohol, to obtain comparable results. This is at variance with the experiences reported above, and is thought by Foss to be due possibly to inefficient inunction of the alcoholic tincture. A decision as to the relative merits of the free or esterified form in tincture was not made. However, Zondek & Sulman [1939] also found that although testosterone in alcohol is superior to testosterone in oil when both are given percutaneously to the castrated rat, it is only about one fourth as effective as testosterone injected in oil.

Scott [1940] compared the activities of methyl testosterone, testosterone and testosterone propionate in castrated rats, inuncting the material in lanol (lanolin) and tegin. Little difference was found between the responses of the prostate and seminal vesicles to the first two (free) compounds, but both were more effective weight for weight than the propionate. Tegin appeared to be a rather better medium than lanol. Nelson, Greene & Wells [1940] also investigated the effectiveness of testosterone and its propionate in castrated rats, using the weight increase in the prostate gland and seminal vesicles as criteria. They found that both compounds were more effective when inuncted in alcohol than in oil, lanolin or 'ointment', and that testosterone was more effective percutaneously in alcohol than subcutaneously in oil. Testosterone propionate,

however, is less effective percutaneously in all media than by injection, and, in general, less effective by inunction than free testosterone. As the volume of media used was 0.1 ml. per treatment, containing 0.2 mg. of androgen, the effectiveness of free testosterone by injection was almost certainly less than would have been found with a larger volume of oil [cf. Deanesly & Parkes, 1936], but the contradictory results of Zondek & Sulman [1939] are not explicable on these grounds, as they also used 0.1 ml. per injection.

As to the mechanism of percutaneous absorption, Lasareff, Brussilovskaya & Livschitz [1931] found that solvents such as benzene, ether and acetone are themselves absorbed into the blood-stream when inuncted on the ear of the rabbit, or on the human skin. They might therefore carry a dissolved hormone with them. Eller & Wolff [1939] also determined permeability of the skin to fats, and found that they penetrate mainly along the hair shafts and oil gland ducts. Liquid fats penetrate more rapidly than solid fats and the depth to which a fat penetrates depends on its nature, being greatest for animal fats, followed by materials of vegetable and then by those of mineral origin. Most fats show an optimum penetration at 4–6 hours after application. A survey of the question is given by Eller & Wolff [1940]. Hydrous wool fat appears only to be absorbed by the keratin layer of the skin [Rothman—quoted by Eller & Wolff, 1940]. Baer [1939] believes, however, that no storage occurs in the skin after the absorption of oestrogens from wool fat, petrolatum or olive oil, as a minimal daily dose is required to maintain oestrus in spayed rats.

The present investigation was undertaken in an attempt to clarify the position with regard to the local and general effectiveness of inuncted sex hormones and their synthetic equivalents. Whilst the previous evidence seemed to show that volatile organic solvents such as benzene or alcohol are unquestionably superior to oils or 'ointments' for the production of an effect in organs remote from the site of inunction, it was not clear whether they have this superiority in producing a strictly local effect. It seemed very possible that, despite Baer's [1939] conclusions, an oily or fatty base retains the active material in the skin for a relatively long period and that a more pronounced local, but a less pronounced systemic activity might be the result. Furthermore, it was thought desirable to determine with some degree of confidence the most useful of several common media for promoting percutaneous absorption.

Esters of the substances investigated have not been used, as it seemed well enough established that they are less effective percutaneously than the free compounds [cf. Moore *et al*, 1938; Scott, 1940; Nelson *et al*, 1940 quoted above].

THE INUNCTION OF OESTROGENS

Spayed mice

Oestrone and diethylstilboestrol were inunctioned in a variety of media on the clipped backs of spayed albino mice (Tables I and II). Each test consisted of two successive daily applications in 0.1 ml. of the medium, which was smeared or poured on the skin and gently massaged for a short period if necessary. The scoring and other details of the tests have been fully described elsewhere [Emmens, 1939]. The vaginal smears which were taken subsequently were marked as positive if cornified or nucleated epithelial cells were present, with no leucocytes.

Table I. *Data relating to the production of vaginal cornification in spayed mice by the inunction of oestrone in various solvents. An application of 0.1 ml. was given on each of two consecutive days.*

Date of test	Solvent	Total dose ($\mu\text{g.}$)	No. of mice	No. +ve.	% +ive response \pm S.E.*
1/4/40	ether	0.075	10	8	80 ± 12.6
"	benzene	0.075	10	6	60 ± 15.5
"	96% alcohol	0.075	8	3	37.5 ± 17.1
"	70% alcohol	0.1	9	3	33.3 ± 15.6
"	nut oil	0.4	8	0	0
"	50% glycerol	0.8	10	9	90 ± 9.5
15/4/40	nut oil	0.8	10	9	90 ± 9.5
20/5/40	lanolin	1.6	10	2	20 ± 12.6
11/6/40	benzene	0.05	10	0	0
"	"	0.075	10	3	30 ± 14.5
"	"	0.1	10	9	90 ± 9.5
1/4/40 to 11/6/40	oil, by injection	0.1	10-20	—	70-90

* S.E. = standard error of mean.

Table II. *Data relating to the production of vaginal cornification in spayed mice by the inunction of diethylstilboestrol in various solvents. An application of 0.1 ml. was given on each of two consecutive days, using 10 mice per group*

Date of test	Solvent	Total dose ($\mu\text{g.}$)	% +ive response \pm S.E.*
20/5/40	ether	0.05	0
"	benzene	"	"
"	96% alcohol	"	"
"	nut oil	0.5	40 ± 15.5
"	50% glycerol	"	70 ± 14.5
"	lanolin	1.0	90 ± 9.5
11/6/40	ether	0.1	50 ± 15.8
"	benzene	"	20 ± 12.6
"	96% alcohol	"	10 ± 9.5
"	oil by injection	"	30 ± 14.5

* S.E. = standard error of mean.

With both oestrogens a solution in ether proved the most efficient, equalling and perhaps exceeding the efficiency of an injected oil solution

in producing vaginal oestrus. Benzene appeared nearly as good a medium as ether, and the responses to equal doses of oestrogen in the two media are not significantly different. 70% or 96% alcohol is significantly less effective than ether ($p < 0.05$ in each case, where p is the probability of the observed differences being due to chance) and more effective than the other solvents used ($p < 0.01$), benzene excepted. Of the remainder, lanolin (anhydrous wool fat) is significantly less effective than nut oil or 50% glycerol in water ($p < 0.01$), which do not differ significantly between themselves. The results with the natural and synthetic oestrogens are completely concordant.

The influence of the solvent on the apparent potency of a given dose of oestrogen is considerable. The potency of oestrone, using the provisional dose/response curve obtained by inunction in benzene on 11/6/40 (Table I) is very approximately 24 with ether, 22 with benzene, 18 with 96% alcohol, 3 with 50% glycerol or nut oil, and 1 with lanolin as the solvent, and 20 for injection in oil. Diethylstilboestrol, showing the same seriation, gives the figures 10 with ether, 8 with benzene or 96% alcohol, 2 with 50% glycerol or nut oil, 1 with lanolin and 8 for injection in oil, the potency by inunction in lanolin being taken as unity in each instance.

These figures reflect the ease with which the two oestrogens are able to reach the vagina in effective quantities and for a sufficient period of time to initiate cornification. Failure to cause cornification might be due to very rapid absorption and elimination, or to inadequate absorption. However, the reactions were not unduly prolonged, as would be expected if lanolin, for instance, retained much of the 1.6 μ g. apparently necessary to cause 20% of cornification and released it over a period of days. The matter seemed amenable to experimentation by inunction to the skin area from which new feathers were growing in cocks or capons, which would show a more intense or prolonged reaction to the locally applied material if it were retained *in situ* by oil or fat. The failure to demonstrate this is recorded in the next section.

Brown Leghorn capons

The breast feathers of groups of Brown Leghorn capons were plucked and the new feathers allowed to grow for 10 days before the application of oestrogen. These feathers, which are black, respond to oestrogenic stimulation by producing salmon-coloured areas which extend along the length of the feather approximately 2 mm. for every 24 hours of adequate stimulation (i.e. the feather grows at approx. 2 mm. per day). Strong stimulation produces a wide, complete bar, and weaker stimulation a rachis stripe only. Deanesly & Parkes [1937] had already shown that 0.25 mg. of oestradiol will produce a weak rachis stripe when inuncted in

oil to the feather tract of Brown Leghorn capons. In the present experiments therefore, 0.5 mg. of oestradiol was applied by a single inunction in different media to the skin area containing growing feathers.

In ether and in 96% alcohol, 0.5 mg. of oestradiol produced, in groups of 5 birds, about equivalent degrees of feminization, varying in different birds from an almost complete bar which narrowed towards the base of the feathers to a central rachis stripe. The visible action of the hormone had continued for between 8 and 16 days, as the central feminized stripe extended for a length of 1.6–3.3 cm. With benzene, a less extensive and rather less prolonged feminization occurred, and an even slighter effect was seen with nut oil. Lanolin as a solvent resulted in a mere trace of feminization on occasional feathers. The same degree of feminization was seen in breast feathers growing at a distance from the inuncted area, and there was thus no strictly local action of oestradiol in any solvent, nor a more prolonged action with those solvents giving a less intensive effect (i.e. a rachis stripe only).

Inunction of diethylstilboestrol produced similar effects, but even 1.5 mg. of the substance did not give extensive feminization. With this compound, 96% alcohol appeared to produce the greatest effect, followed by ether and benzene. In lanolin and nut-oil, it gave faint occasional traces of fawn colouration. This ineffectiveness of inuncted diethylstilboestrol reflects that already reported with regard to its anti-androgenic action when inuncted on the capon comb [Emmens & Bradshaw, 1939]. The data now presented seem to show that the inefficiency is due to inadequate absorption, as a long rachis stripe may be produced, showing that the substance has not been rapidly absorbed and eliminated.

THE INUNCTION OF ANDROGENS

Castrated male rats

Thirty young male albino rats were castrated and rested for 5 weeks, and then inuncted for 10 days on the shaved flanks with 0.5 mg. per day of testosterone in 0.1 ml. of various solvents. One group was injected with the same dose of hormone in 0.5 ml. of nut oil per day. The prostate gland and seminal vesicles were then dissected and prepared as described by Callow & Deanesly [1935]. The results are shown in Table III.

The average weight of the prostate gland of rats inuncted with the hormone in benzene or 96% alcohol was significantly greater than that when ether was the medium ($P < 0.02$), which in its turn was superior to lanolin ($P < 0.02$), which was superior to nut oil ($P < 0.01$). Inunction in benzene proved better than injection in oil ($P < 0.05$). The probabilities are those found with Fisher's [1938] *t* test.

Table III. *The weight of the prostate gland and seminal vesicles of castrated male rats following the daily inunction of 0.5 mg. of testosterone in 0.1 ml. of various solvents for 10 days. All tests, on 5 rats per group, were made simultaneously*

Solvent	Av. body-wt. (g.)	Av. wt. of prostate gland ±S.E.* (mg.)	Av. wt. of seminal vesicles ±S.E.* (mg.)
Benzene	171	258 ± 5.8	153 ± 20.1
96% alcohol	179	236 ± 20.0	115 ± 12.4
Ether	167	184 ± 10.5	95 ± 5.6
Lanolin	197	136 ± 6.4	46 ± 4.3
Oil	169	103 ± 6.6	33 ± 2.9
Oil by injection (0.5 ml. daily)	180	195 ± 23.2	80 ± 11.4

* S.E. = standard error of mean.

The same order of apparent potency was shown by the seminal vesicle weights, with benzene > ether ($P < 0.01$) > lanolin ($P < 0.01$) > oil ($P < 0.05$). The seminal vesicle weights with 96% alcohol did not differ significantly from those with benzene or ether. Once more, inunction in benzene was superior to injection in oil.

Brown Leghorn capons

As a corollary to the tests with castrated rats, a further series of simultaneous tests was run on groups of 9 or 10 Brown Leghorn capons by inunction on the plucked breast. Response was measured as the added increases in length and height of the comb which were produced by treatment [cf. Emmens, 1939]. Three consecutive daily inunctions of 40 μ g. of testosterone in 1 ml. of medium were given in ether, benzene and 96% alcohol, and three corresponding injections of 40 μ g. in 0.1 ml. of nut oil. The daily amount of testosterone was raised to 200 μ g. for lanolin and oil by injection.

Table IV. *The maximum comb growth (increase in length plus height) produced by three daily inunctions of testosterone in 1 ml. of various media on the plucked breast of Brown Leghorn capons. All tests were conducted simultaneously*

Solvent	Total dose (mg.)	No. of birds	Av. comb growth ± S.E.* (mm.)
Benzene	0.12	10	2.20 ± 0.33
Ether	0.12	9	1.70 ± 0.30
96% alcohol	0.12	10	1.30 ± 0.26
Lanolin	0.60	9	3.15 ± 0.27
Oil	0.60	10	2.40 ± 0.46
Oil by injection	0.12	10	3.75 ± 0.45

* S.E. = standard error of mean.

The results (Table IV) show that injection in oil was superior to all inunctions, the comb growth produced being significantly greater than

with inunction in benzene ($P < 0.02$). Inunction in benzene gave significantly more growth than in 96% alcohol (P c. 0.05), that in ether falling intermediately. The three volatile organic solvents were once more clearly superior to oil and lanolin, which, with 5 times the dose of testosterone, produced only slightly more comb growth.

The comb growth produced by inuncted testosterone in all of the media tested did not reach a maximum until 4 days after the last inunction, and had commenced to decrease by the 7th day. That resulting from injection in oil, however, was already falling on the 4th day after the last injection.

The Brown Leghorn capon comb

The local effectiveness of different solvents for androgens may be tested by direct inunction on the comb. By this method, introduced by Fussgänger [1934] a very small quantity of androsterone will produce measurable comb growth. Emmens [1939] had shown that 3 μ g. of androsterone inuncted in oil will produce an increase in length plus height of 3–5 mm., hence this quantity was given in different media by three daily applications of 1 μ g. in 0.1 ml. of solvent. All tests were carried out together, with 10 birds per group. The results will be examined in greater detail in a subsequent communication, those given in Table V being sufficient for the present purpose. The mean comb-growth on the day after the last inunction is given in the table, although the combs continued to grow for several days beyond this. Combs inuncted with androsterone in lanolin, oil, and benzene did not increase in size after the 4th day following the last inunction, but those inuncted with 96% alcohol and ether continued to grow slightly until the 7th day. The order of activity in the different solvents was not affected by this added growth, which in no case exceeded 45% of the figures given in Table V. Once more we see that the relatively inefficient solvents do not produce delayed absorption, which should result in more prolonged comb growth compared with the others.

Table V. *Comb growth (increase in length plus height) produced by the direct inunction of 3 μ g. of androsterone in various media on the combs of Brown Leghorn capons. All tests were carried out simultaneously with 10 birds per group*

Solvent	Av. comb growth \pm S.E.* (mm.)
Alcohol	4.95 \pm 0.72
Benzene	4.70 \pm 0.45
Ether	3.75 \pm 0.37
Oil	3.20 \pm 0.72
Lanolin	2.95 \pm 0.40

* S.E. = standard error of mean

The comb growth resulting from inunction in 96% alcohol, benzene, oil and ether did not vary significantly, and only alcohol and benzene were significantly superior to lanolin ($p < 0.05$ and *c.* 0.01 resp.). There was thus little difference in action between the various solvents when applied directly to the site of action, the comb.

INUNCTION OF PROGESTERONE ON RABBITS

McPhail tests [1934] for progestational activity were conducted with Dutch rabbits, five daily inunctions in 0.2 ml. of medium being given on the shaved flanks. The results were scored as by Emmens & Parkes [1939], giving marks of $\frac{1}{2}$ –4 according to the degree of proliferation, and are shown in Table VI.

The scores with benzene were significantly higher than those with 96% alcohol ($p < 0.01$), which were in turn significantly higher than those with ether or oil ($p < 0.05$). Ether was not significantly superior to oil in these tests, a rather surprising result. Inunction in benzene is as effective as injection in oil, the response to a total of 0.5 mg. by injection being between 2 and 3.

Table VI. *The response of the rabbit uterus to progesterone inuncted in various media on the shaved skin by five daily applications each of 0.2 ml. with 3 animals per group*

Solvent	Total dose (mg.)	Mean response \pm S.E.*
Benzene	0.5	2.3 ± 0.18
	1.0	2.3 ± 0.21
	2.0	3.2 ± 0.18
96% alcohol	0.5	0.8 ± 0.33
	1.0	1.7 ± 0.60
	2.0	2.0 ± 0.29
Ether	0.5	1.0 ± 0.74
	1.0	0.5 ± 0.29
	2.0	0.5 ± 0.29
Oil	0.5	0.2 ± 0.18
	1.0	0.5 ± 0.00
	2.0	0.5 ± 0.29

* S.E. = standard error of mean.

DISCUSSION

The results of this investigation have fully confirmed those of other workers, in showing that the volatile organic solvents are superior to oil or fat in promoting the percutaneous absorption of sex hormones. There seems little to choose between ether, benzene and 96% alcohol, but ether has headed the list in each test with oestrogens, and benzene in all but one with androgens, being not significantly worse than alcohol in the

tests involving local application to the capon comb. Benzene was also superior to the others in progestational tests with rabbits. Similarly, whereas oil has been placed last in each series of tests with androgens and progesterone, but not always significantly worse than lanolin, lanolin has proved least effective in each test with oestrogens. There thus appears to be a slight but consistent difference between the effectiveness of different media according to the group of steroid hormones which is being tested, diethylstilboestrol agreeing in general with oestradiol and oestrone.

Large differences have been found in the apparent potency of both oestrogens and androgens when inuncted in different media, in tests involving action at a distance from the site of inunction, such as vaginal cornification in mice and prostate growth in rats. Smaller differences, often not significant, were found when the object of the test was to assess the local effectiveness of the media in the capon comb-growth test, and although the tests involving the inunction of oestrogens on growing feather-tracts showed well-marked differences between media, the action was not found to be strictly local.

A further result of the tests has been an apparent negation of the idea that an oily or fatty base promotes retention of material and prolongs either its local or systemic action, as compared with other solvents. Nut oil and lanolin were relatively inefficient, whether intensity or duration of action was considered, locally or systemically. Whilst direct inunction of active material on the test-object is very effective in the case of the capon comb, this effectiveness is little influenced by the medium.

On the other hand, inunction in all of the media tested was followed by some delay in absorption, a delay of a few days when the activity of the hormone was measured by test-objects distant from the site of inunction, and of one or two weeks when its local effects were observed. This tendency was, however, more marked with the volatile solvents than with oil or lanolin. Such a delay, when added to that caused by esterification, explains why the inunction of esters is not very efficient when compared with injection, as too great a slowing of absorption may occur.

A very pertinent question would appear to be that concerning the fate of material inuncted in oil or fat. If the visible action of oestradiol when applied to the skin containing growing feathers is neither as intense nor as prolonged dose for dose in lanolin as in ether, what happens to the oestrogen? It seems unlikely that it is very slowly absorbed, since the rachis stripe produced should, under those conditions, be longer than that produced with the more effective solvents, and the comb growth from inuncted androgen should continue longer with oil or lanolin than with ether, but it does not, whether the inunction is made locally on the comb or on the skin. One must suppose that destruction or elimination of the

active material may be facilitated when it is applied in oil or fat, but in view of Foss's findings, quoted above, that an ointment containing testosterone is more efficient in man than is an alcoholic tincture, the question must be regarded as unclarified. It certainly seems probable that a volatile solvent leaves behind it a precipitate of the hormone in or on the skin, which would seem a very effective method of administration, and that oil or fat does not.

SUMMARY

1. Various androgens and oestrogens and progesterone have been injected in different media on the skin of mammals and capons, and on the capon comb. Their systemic activity has been measured by the production of vaginal cornification in spayed mice, enlargement of the prostate gland and seminal vesicles in castrated rats, progestational proliferation in rabbits and the growth of the comb in capons. Their local activity has been measured by feather reactions and comb growth when applied to the site of action in capons.

2. The tests have agreed in showing that, whether local or systemic activity is considered, the volatile organic solvents, ether, benzene and 96% alcohol, are more effective as carriers of active material from the skin surface than is oil or lanolin. In tests with rats and mice, injection in benzene or ether is as effective as or a little better than injection in oil. In tests with rabbits, injection in benzene, but not in ether, is superior to injection in oil.

3. A slight but consistent superiority of ether as a medium was found when dealing with oestrogens, and of benzene when dealing with androgens and progesterone.

The testosterone and oestradiol used in these investigations were generously provided by Messrs. Ciba Ltd. & Organon Laboratories respectively.

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COMPARATIVE ACTIVITY OF THE GONADOTROPHIN IN HORSE PITUITARY GLANDS AND IN PREGNANT MARES' SERUM

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NOBLE, Rowlands, Warwick & Williams [1939] studied the histological effects exerted on the ovaries of hypophysectomized rats by gonadotrophic extracts from different sources, viz.: the pituitary glands of horses, oxen, sheep and pigs, the serum of pregnant mares and the urine of pregnant women. Of these extracts only those prepared from horse pituitary gland stimulated the ovary of the hypophysectomized rat in such a way that it resembled that of a normal adult rat. It was observed, also, that extracts prepared from the serum of pregnant mares had certain properties similar to those of extracts of horse pituitary gland, but that differences occurred which seemed related to the degree of luteinization caused. In view, therefore, of the general biological interest of these two types of gonadotrophic preparation obtained from the same species and also because of their possible therapeutic use it seemed desirable to us that their comparative activities should be investigated in greater detail.

On account of their greater availability extracts of pregnant mares' serum have been studied by previous workers more extensively than preparations obtained from the pituitary gland of horses. Observations have been made on the effects as seen in a large number of animal species, but usually in animals with their pituitary gland intact. It is generally agreed that ovulation can be caused in such animals by injection of extracts of pregnant mares' serum. Ovulation was readily induced in the rat [Cartland & Nelson, 1938] and in man [Davis & Koff, 1938], but less readily in the anoestrous cat [Windle, 1939] and in monkeys having non-ovulatory menstrual cycles [Hartman, 1938].

The quantitative response of the ovaries of hypophysectomized rats to extracts of pregnant mares' serum has been studied by Pencharz [1939], Leatham [1939] and Pencharz, Cole & Goss [1940]. Leatham reported that corpora lutea are formed in the ovaries of the animals but it is not stated whether ovulation occurred. We have been unable, with one exception, to cause ovulation in the hypophysectomized rat with pregnant mares' serum. On the other hand, we have confirmed our

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previous finding that ovulation can be produced in similar test animals by the injection of an extract of horse pituitary gland. It is in this respect that we observed one of the major differences between extracts from these two sources.

MATERIAL AND METHODS

Extracts

Two extracts were prepared from horse pituitary glands: (a) AP61B, an alkaline aqueous extract prepared from acetone-desiccated anterior pituitary glands of mares and stallions, and (b) AP70B, an extract similarly prepared from the dried glands of geldings. One mg. of each extract is equivalent to about 20 mg. of the fresh glands.

Several extracts of pregnant mares' serum were tested. These include: (a) PMS3, PMS13, PMS16 and PMS18, all specimens of the commercial preparation 'Antex'; (b) MS1, MS2+7, MS4 and MS6, specimens of contributions towards the International Standard preparation of mare serum gonadotrophin, which were circulated for comparative trials under the auspices of the Health Organization of the League of Nations; (c) the International Standard; (d) PMS26, unextracted serum which was supplied by the Wellcome Physiological Research Laboratories, and (e) serum samples A₃ and A₄ [see Day & Rowlands, 1940].

Biological assay

The extracts were assayed by their capacity to increase the weight of the ovaries of immature rats and to cause ovulation in oestrous rabbits.

Intact immature rats

Groups of 5 or more immature female rats weighing 40–50 g. were injected subcutaneously once daily on five consecutive days, or the total dose was given in one injection on the first day. They were killed on the 6th day and the ovaries and uterus were dissected. These organs were fixed in Bouin's fluid overnight and then weighed after immersion for a few hours in 70% alcohol.

Hypophysectomized immature rats

Similar rats were hypophysectomized and 10–12 days later were injected as described above. Figures obtained from incompletely hypophysectomized animals, detected by examination of the sella turcica at autopsy, were excluded from the records.

Oestrous rabbits

Groups of 10 oestrous rabbits were given a single intravenous injection into the marginal ear vein and the ovaries inspected at laparotomy 24

hours later. The results are expressed as the percentage number of animals in which ovulation was found.

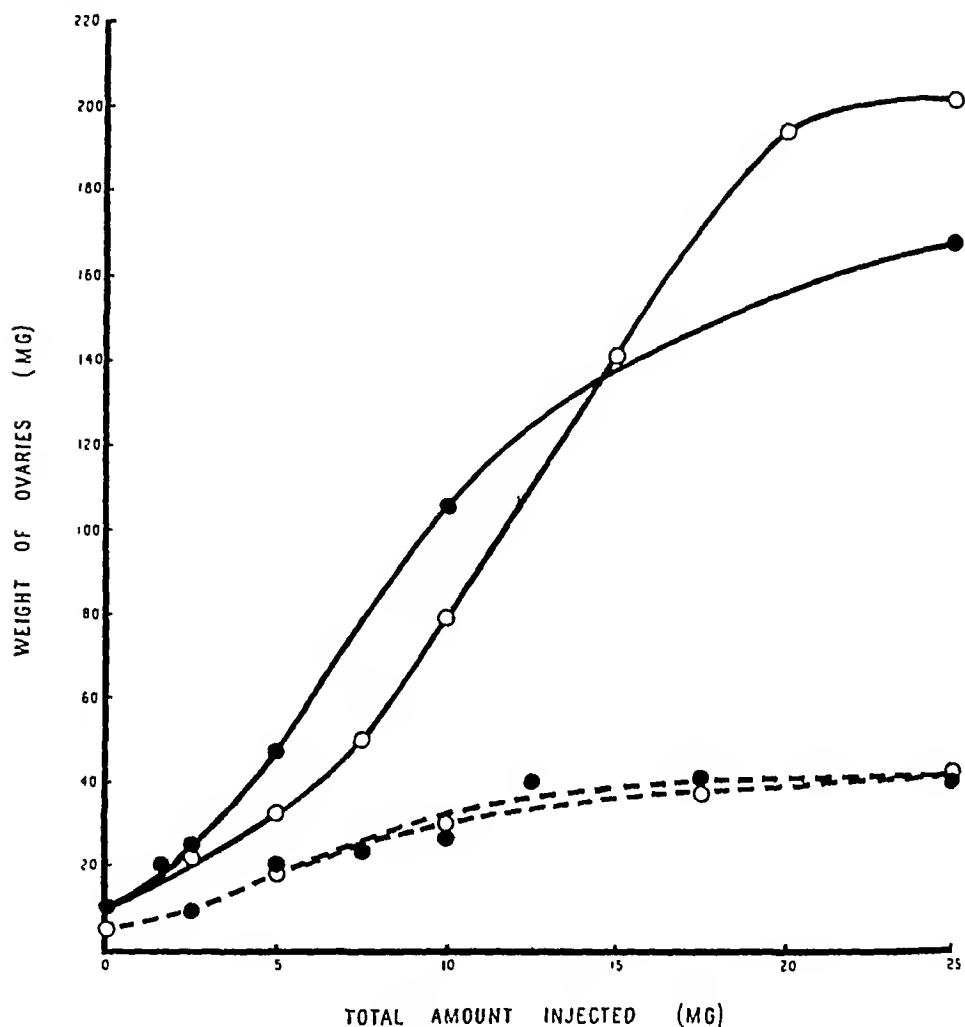


FIG. 1. The effect of extracts of pregnant mares' serum on the ovaries of intact and hypophysectomized rats.

- = International Standard into intact rats.
- = International Standard into hypophysectomized rats.
- = MS1 into intact rats.
- = MS1 into hypophysectomized rats.

Histology

The ovaries and uteri of the rats were prepared for histological examination in the usual way; the ovaries were cut serially, every fifth section being mounted and stained with haematoxylin and eosin. The Fallopian tubes of some of the rats were cut serially, all the sections being mounted so as to facilitate examination for tubal ova.

RESULTS

Quantitative differences in responses of ovaries of intact rats to extracts of horse pituitary glands and of pregnant mares' serum

Differences in response to five daily injections

The nature of the quantitative response of the ovaries of intact rats to these extracts by this method of assay has recently been examined by

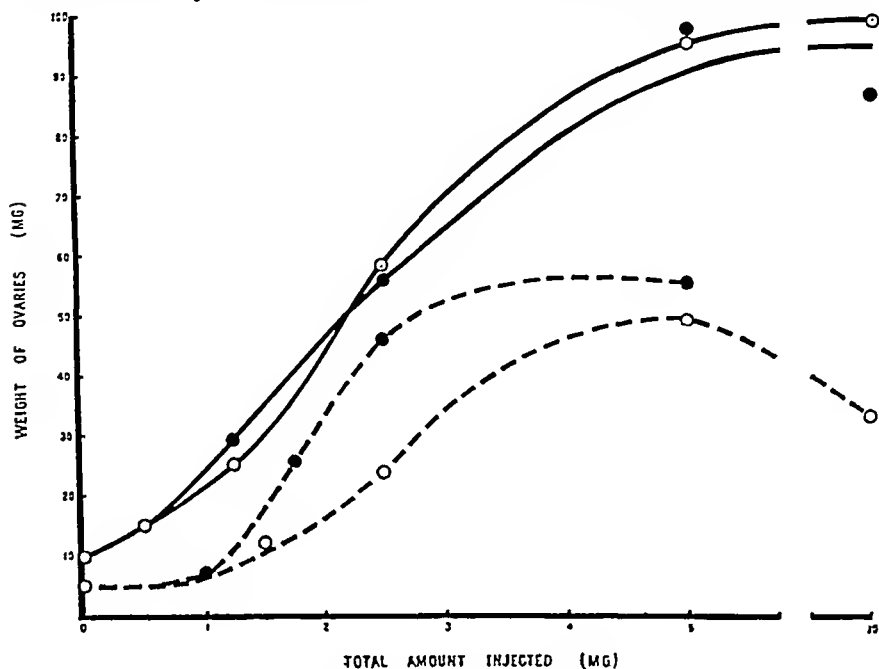


FIG. 2. The effect of two extracts of horse pituitary glands on the ovaries of intact and hypophysectomized rats.

- = AP61B into intact rats.
- = AP61B into hypophysectomized rats.
- = AP70B into intact rats.
- = AP70B into hypophysectomized rats.

Emmens [1940]. The main difference observed was that the average maximum response obtained with extracts of pregnant mares' serum was about twice as great as that given by extracts of horse pituitary gland. This difference is illustrated in Figs. 1 and 2 which show dose-response curves for the weight of the ovaries of intact and hypophysectomized rats injected respectively with two preparations of mare serum gonadotrophin (MS1 and International Standard) and two extracts of horse pituitary gland (AP61B and AP70B). The full data relating to these results are given in Tables I and II. It is seen that in intact rats the response to the

Table I. *Assay of extracts of pregnant mares' serum in intact and hypophysectomized rats*

Extract		Assay in intact rats			Assay in hypophysectomized rats			
No.	Total amount injected (mg.)	No. of rats	Body-weight (g.)	Weight of ovaries (mg.)	No. of rats	Body-weight (g.)	Weight of ovaries (mg.)	Qualitative response of ovaries
MS1	0	10	50	10	4	50	5	Atrophic
"	1.5	10	51	20	—	—	—	
"	2.5	10	48	25	—	—	—	
"	5.0	10	47	47	6	50	18	I.C.S. Hypoœmia
"	10.0	10	49	106	6	55	30	F.S. (slight) in 3 F.S.—M.G.L. in 3 } I.C.S.
"	17.5	—	—	—	6	56	47	F.S. with M.G.L. and I.C.S.
"	25.0	10	44	168	5	58	52	F.S. with M.G.L. and I.C.S.
MS4	0.15	10	50	33	—	—	—	
"	0.325	—	—	—	6	53	41	F.S. no M.G.L. I.C.S.
MS6	1.5	9	45	45	—	—	—	
"	3.25	—	—	—	6	49	32	F.S. no M.G.L. I.C.S.
MS7+2	2.5	10	50	21	—	—	—	
"	10.0	—	—	—	5	50	37	F.S. with slight M.G.L. I.C.S.
MS Standard	2.5	10	54	22	4	45	9	I.C.S.
"	5.0	10	53	33	5	50	20	Slight F.S. in 3 I.C.S.
"	7.5	10	46	50	5	49	23	Slight F.S. I.C.S.
"	10.0	10	44	79	4	50	26	
"	12.5	—	—	—	6	47	40	F.S. with some M.G.L. I.C.S.
"	15.0	10	47	141	—	—	—	
"	17.5	—	—	—	5	56	41	
"	20.0	10	50	194	—	—	—	
"	25.0	10	51	202	5	47	40	F.S. with M.G.L. I.C.S.

F.S. = follicle stimulation
 I.C.S. = interstitial cell stimulation
 C.L. = corpora lutea
 M.G.L. = luteinization of membrana granulosa in the absence of ovulation.

serum preparations becomes asymptotic at about 200 mg., which is in close agreement with the results of Cartland & Nelson [1938]. In hypophysectomized rats, however, the average maximum ovarian weight obtained with these substances is only about 40 mg. Pencharz [1939] who compared the effectiveness of pregnant mares' serum in normal and hypophysectomized rats found that when small amounts were given the response was two to three times greater in normal rats, but that larger amounts caused an approximately equal response in both types of rats,

Table II. *Assay of extract of horse (AP61B) and gelding (AP70B) pituitary glands in intact and hypophysectomized rats*

No. (AP)	Extract	Assay in intact rats			Assay in hypophysectomized rats			Qualitative response of ovaries
	Total amount injected (mg.)	No. of rats	Body- weight (g.)	Weight of ovaries (mg.)	No. of rats	Body- weight (g.)	Weight of ovaries (mg.)	
61B	0	10	50	10	4	50	5	Atrophic
"	1.25	10	47	25	—	—	—	
"	1.5	—	—	—	5	55	12	Slight F.S. and some I.C.S.
"	2.5	10	51	58	4	50	24	F.S. I.C.S. M.G.L. and C.L. in 1 ovary
"	5.0	10	53	95	10	58	47	F.S. M.G.L. and C.L.
"	10.0	5	49	99	6	48	34	F.S. M.G.L. Small solid C.L. with ova
70B	1.0	—	—	—	4	45	7	Slight F.S. and I.C.S. in 1 pair of ovaries
"	1.25	5	52	29	—	—	—	
"	1.75	—	—	—	10	50	26	F.S. and C.L. M.G.L. and slight I.C.S.
"	2.5	10	54	56	14	54	46	F.S. with M.G.L. and C.L.
"	5.0	5	52	97	5	56	55	F.S. with M.G.L. and C.L.
"	10.0	10	50	86	—	—	—	

F.S. = follicle stimulation

I.C.S. = interstitial cell stimulation

C.L. = corpora lutea

M.G.L. = luteinization of membrana granulosa in the absence of ovulation.

which indicates that ovaries weighing 250 mg. were produced in a 4-day period. Leathem [1939], too, has recorded ovarian weights of 180 mg. in hypophysectomized rats treated with similar substances. While we allow an interval of from 10 to 12 days to elapse between operation and assay the corresponding intervals allowed by Leathem and Pencharz were 6 and 8 days respectively. It is very doubtful whether the failure of the mare serum preparations used by us to increase the weight of the ovaries above 40 mg. is caused by this longer interval.

Fig. 2 shows that the response of intact rats to extracts from the pituitary glands of mares and stallions or of geldings, reaches a maximum at 100 mg. and that the corresponding response in hypophysectomized rats is 50 mg. In intact rats the activity of the two extracts is very similar, but in hypophysectomized rats, where the measure of activity is not complicated by the secretion of endogenous gonadotrophin, the gelding

pituitary extract is considerably more active in small amounts although the maximum response is the same in each case.

In the intact rat, therefore, extracts of pregnant mares' serum produce a maximum response which is twice as great as that caused by horse pituitary gland. On the other hand, the maximum response produced by pregnant mares' serum in hypophysectomized rats is slightly less than that which occurs in similar rats when treated with horse pituitary gland.

Assays which have been carried out subsequently indicate that the sensitivity of the test animal to the injection of the International Standard of mare serum gonadotrophin has altered. The original dose-response curve, shown in Fig. 1, was constructed during November 1939. Similar assays carried out in July 1940 indicate that the sensitivity of the rat had increased to such an extent that the response given by a series of different doses has in each case increased by about 25%. It is not possible at the present time to say whether this change in sensitivity is seasonal or not, but it is proposed to continue the investigations into this problem.

Differences in response to single injections

In the paper by Cole & Hart [1930] in which they originally reported the occurrence of a gonadotrophic principle in the serum of pregnant mares they observed that the response caused by a single injection of the serum was similar to that resulting from a number of divided doses. The response, therefore, is, within limits, independent of the degree of subdivision of the total dosage. This result was subsequently confirmed by Cole, Guilbert & Goss [1932], Hamburger & Pedersen-Bjergaard [1938] and Deanesly [1939].

The quantitative response of the ovaries of intact rats to MS1 [see Deanesly, 1939] was the same when given in one single injection as it was when the total dosage was divided and given in five equal daily injections, the animals in each group being killed on the 6th day. The response to a similar dose in one injection at the end of the three days is, however, as shown in Table III, considerably less, which indicates that the animal utilizes and responds to the injected substance until at least the fifth day. Another experiment, using PMS26 and hypophysectomized rats, gave a similar result.

In contrast, Deanesly [1939] has shown that with injection into intact rats, 2.5 mg. of the extract of horse pituitary gland, AP61B, an amount which by the usual method of assay produces ovaries weighing 55 mg., is almost ineffective when it is all injected in one dose, the animals being killed for examination five days later.

The difference in the duration of action between extracts from these two sources is probably caused by the greater size of molecule to which

the hormone in the serum is attached. Evidence favouring this is: (a) that the hormone in the serum is neither dialysable nor ultrafiltrable [Goss & Cole, 1931], and (b) that it is not excreted by the kidneys into the urine of the mare. In this respect the gonadotrophin in pregnant mares' serum differs from that which occurs in the serum of the pregnant woman, which is not retained by the human kidney. This difference is demonstrated by the results given in Table III, which show that the response in the rat ovary to the hormone in human pregnancy serum is dependent on the degree of subdivision of the dose.

Table III. *Duration of action of extracts of the serum of pregnant women and mares, and of extracts of horse pituitary glands*

Extract	No. of rats	Method of administration		No. of days injected	Time of killing after 1st injection (days)	Body- weight (g.)	Weight of ovaries (mg.)	Weight of uterus (mg.)
		Amount in mg.						
		Daily	Total					
MS1	10	1.0	5.0	5	5	47	47	99
"	10	5.0	5.0	1	3	53	30	74
"	10	5.0	5.0	1	5	48	48	81
"	5	1.5	7.5	5	5	56	62	90
"	5	7.5	7.5	1	5	59	77	86
AP61B	5	0.5	2.5	5	5	55	55	100
"	5	2.5	2.5	1	5	53	13	64
PMS26	10	0.1 ml.	0.5	5	5	50	203	80
"	6*	0.1 ml.	0.5	5	5	53	45	83
"	7*	0.5 ml.	0.5	1	5	56	54	123
PWS77†	10	0.1	0.5	5	5	58	31	77
"	10	0.5	0.5	1	5	50	18	50

* = Hypophysectomized rats.

† PWS77 = an extract prepared from the serum of pregnant women.

Qualitative differences in responses of ovaries of hypophysectomized rats to extracts of pregnant mares' serum and of horse pituitary glands

In the previous section it has been shown that the response of the ovaries of intact rats to extracts possessing gonadotrophic activity is different from that in hypophysectomized rats, probably owing to the intervention of endogenous gonadotrophic substances present in the test animal. It is essential, therefore, that the histological nature of the direct response of the ovaries to the extracts themselves should be determined by tests on hypophysectomized animals. We have examined, by these means, the effect of several extracts prepared from pregnant mares' serum, including MS1, MS4, MS6, MS2+7 and the International Standard, and also two samples of serum which were not treated except for drying in high vacuo at low temperature [Greaves & Adair, 1938].

Their action is compared with that of the extracts of horse pituitary gland (AP61B) and of gelding pituitary gland (AP70B).

Histological response of the ovary of the hypophysectomized rat

Small doses of pregnant mares' serum which increase the weight of the ovaries to about 20 mg. cause little, if any, follicle stimulation. The primary, direct effect of pregnant mares' serum is, as has been pointed out by Leatham [1939], to increase the interstitial tissue and to cause luteinization of the theca and membrana granulosa of atrophic follicles. The action of small doses of this substance is, therefore, similar to that obtained with chorionic gonadotrophin (human urine of pregnancy). Larger doses of pregnant mares' serum, however, cause follicle stimulation (see Table I). Over-stimulation gives rise to cystic follicles and, less frequently, to follicles whose membrana granulosa becomes luteinized in the absence of ovulation.

The histological response of the ovaries of hypophysectomized rats treated with the preparations of pregnant mares' serum offered as contributions to the International Standard is so strikingly similar that it can be said that the constituents of the standard preparation are qualitatively constant. As with other preparations of pregnant mares' serum when injected in adequate doses, all the contributions in question cause follicular development in the ovaries of hypophysectomized rats but little luteinization of the membrana granulosa. As will be shown later, a mixture of these extracts (the International Standard itself) is unable to cause ovulation in hypophysectomized rats.

The qualitative response of the ovaries of hypophysectomized rats to extracts of horse and gelding pituitary glands is shown in Table II, which indicates that small amounts of these substances, in comparison with extracts of pregnant mares' serum, produce more follicular and less interstitial stimulation. Ovaries stimulated with horse pituitary gland to a weight of about 25 mg. contain numerous mature follicles which may (a) become cystic with degeneration of the membrana granulosa, (b) luteinize in the absence of ovulation, and (c) ovulate and form normal corpora lutea. The occurrence of ovulation is dealt with later. Larger amounts of these extracts cause the development of follicles which luteinize precociously and form masses of luteinized tissue resembling small corpora lutea. These structures resemble those produced in the ovary of the hypophysectomized rat treated with either pig or sheep pituitary extract [Noble *et al.*, 1939]. The presence of these small 'corpora lutea' probably accounts for the decreased quantitative response (see Fig. 2 and Table II) and suggests that the extra luteinizing hormone present in large doses tends to inhibit normal follicular development.

Occurrence of ovulation

The occurrence of ovulation was determined by examination of the Fallopian tubes, in serial section, for the presence of ova. The results are given in Table IV. The extract of horse pituitary gland, AP61B, causes ovulation in both intact and hypophysectomized rats, although larger amounts are required in the latter test animal. The data, though not very extensive, indicate that an optimal dose is necessary to produce ovulation, a result which is in keeping with the histological findings described and with Cole's [1936] observations on intact rats treated with mare serum gonadotrophin. Cole also observed that a larger number of follicles ovulated when the period between injection and autopsy was increased.

Table IV. *Comparison of the ovulation-producing capacity of extracts prepared from horse pituitary glands and pregnant mares' serum*

Gonadotrophic substance			Test rats		Weight of ovaries (mg.)	No. of rats ovulated	Total no. of ova in tubes	Per-centage ovulated
Source	No.	Amount injected (mg.)	Type	No.				
Horse pituitary gland	AP61B	2.5	I*	8	56	8	66	100
	"	5.0	H†	10	47	5	30	50
	"	10.0	H	5	33	0	0	0
Gelding pituitary gland	AP70B	2.5	I	9	59	4	16	45
	"	2.5	H	4	34	1	2	25
Pregnant mares' serum	Mare A 3	0.25 ml.	H	5	39	0	0	0
	Mare A 4	0.5 ml.	H	4	47	1	5	25
	Inter-national	5.0	I	10	39	2	5	20
	Standard	10.0	I	10	92	2	8	20
	Preparation	10.0	H	4	26	0	0	0
		17.5	H	5	41	0	0	0

* I = Intact immature rat.

† H = Hypophysectomized immature rat.

Extracts of pregnant mares' serum, on the other hand, with one exception, failed to cause ovulation in the hypophysectomized rat although these same extracts were effective in intact rats. This result also agrees with the histological findings observed in the ovaries of the hypophysectomized rats where the absence of corpora lutea almost certainly precludes the possibility of the ova having passed into the uterus during the 5-day test period. Confirmation of this was obtained from two assays in which the duration of the test was reduced to 2 and 3 days respectively. In rats treated with mare serum extracts, therefore, the pituitary gland is essential for the occurrence of ovulation. The data indicate also that ovulation is achieved less readily, even in intact rats, with extracts of pregnant mares' serum than with those of horse pituitary glands.

Comparative activity of extracts of pregnant mares' serum and of horse pituitary glands in immature rats and in oestrous rabbits

As the response of the ovaries of the immature rat depends on both follicle-stimulating and luteinizing hormones, which are present in pregnant mares' serum and horse pituitary gland in different proportions, it is not possible by use of this response to standardize either of these extracts in terms of the other and, in particular, extracts of horse pituitary gland cannot be assayed in terms of the International Standard for mare serum gonadotrophin. However, to make a rough quantitative comparison between the extracts we have calculated the amount of each substance required to produce (a) an ovarian weight of 30 mg. in intact rats, and (b) ovulation in 50% of a group of oestrous rabbits. The results are given in Table V.

Table V. *Comparative activity of pregnant mares' serum and horse pituitary glands in intact rats and in oestrous rabbits*

Source of extract	No. of extract	Amount required to produce ovaries weighing 30 mg. in intact immature rat (mg.)	Amount required to cause ovulation in 50% of a group of rabbits (mg.)
Horse pituitary gland	AP61B	1.4	0.25
Golding pituitary gland	AP70B	1.25	0.35
	International Standard	4.5	5.5
	MS1	3.0	2.4
	MS4	0.15	0.18
	MS6	1.0	1.2
Pregnant mares' serum	MS7+2	4.0	5.3
	PMS3	1.5	0.5
	PMS12	2.0	2.1
	PMS13	3.0	2.4
	PMS16	0.5	0.5
	PMS18	0.45	0.2

Judged by the above criteria, it is seen that the relative gonadotrophic activities of the various specimens of pregnant mares' serum in the intact rat are closely paralleled (with two exceptions—PMS3 and PMS18) by their relative activities in the oestrous rabbit. One of the two exceptions (PMS3), a sample of 'Antex' (Løvens Kemiske Fabrik, Copenhagen), was stated by the manufacturers to contain unusually large amounts of luteinizing hormone. If, as it seems probable, this hormone is responsible for the production of ovulation, then this statement by the manufacturers is fully confirmed. Further evidence for the part played by the luteinizing hormone in causing ovulation is given by the data for the extracts of horse pituitary glands. Whereas the glandular extracts are only about three

times as active as the International Standard for mare serum gonadotrophin in stimulating ovarian growth in the intact rat, they are approximately twenty times as active in producing ovulation in the oestrous rabbit.

DISCUSSION

The observations recorded above, combined with those of Noble *et al.* [1939], Boycott & Rowlands [1938], Emmens [1940], Rowlands & Sharpey-Schafer [1940] and Williams [1940] enable us to give some account of the mechanism of action of gonadotrophic substances on the ovary. Observations have been made on both the maximum quantitative response and the qualitative nature of the response of the ovaries of intact and hypophysectomized rats. To account for the multiplicity of the types of both quantitative and qualitative response obtainable with gonadotrophic extracts from various sources we find it necessary to assume the existence of two active principles: (a) the follicle-stimulating hormone and (b) the luteinizing hormone which are present in different proportions in the extracts.

Table VI gives the approximate maximum weight of the ovaries of intact and hypophysectomized rats obtainable with gonadotrophic extracts prepared from various sources.

Table VI. *Maximum ovarian weights of intact and hypophysectomized rats treated with gonadotrophins from various sources*

Extract	Maximum ovarian weight in mg.	
	Intact rat	Hypophysectomized rat
Pregnant mares' serum	200-220	40-50
Horse pituitary gland	100	60
Human pituitary gland	100	70-80
Pregnant women's serum	60	10
Pregnant women's urine	40	10
Sheep } pituitary gland	30	25
Pig }		
Ox pituitary gland	15	10

The maximum ovarian weight produced by each of the above extracts is less in the hypophysectomized rat than in the intact rat. The greater response in the intact rat was attributed previously to the co-operative action of endogenous gonadotrophin secreted by the test animal's own pituitary gland and that only when this latter stimulation is removed, such as after hypophysectomy, is the true gonadotrophic activity of the extract itself revealed. Recently, however, one of us [Williams, 1940] working independently, has shown that the response (increase in weight) of the ovaries of hypophysectomized rats to the injection of gonadotrophin from pregnant mares' serum is greatly increased by previous treatment

of the animal with oestrogen. This increase is caused by the luteinization of all the follicles which were by these means converted into corpora lutea in the absence of ovulation. The oestrogen used (diethylstilbestrol) prevented the atrophic changes and decrease in weight of the ovary which normally occurs after hypophysectomy. No other effect of the oestrogen on the ovary was observed. Luteinization was absent [Williams, 1941].

Although one cannot exclude entirely the influence of endogenous gonadotrophin on the response of the ovary of the intact rat to injections of various gonadotrophic extracts (there is evidence that oestrogens by stimulating the pituitary gland produce luteinization of the ovarian follicles) it seems more probable that the greater effectiveness of the extracts in the intact animal is caused by the condition of the ovaries at the beginning of the test period.

The maximum response of the ovaries of intact rats to extracts of horse pituitary gland is only about one half that produced by mare serum gonadotrophin, yet in hypophysectomized rats it is more than equal. It seems likely that this paradoxical result is caused by a higher ratio of luteinizing to follicle-stimulating hormone in horse pituitary gland. When this proportion is increased further, such as in sheep, pig and ox pituitary gland the maximum ovarian weight obtainable becomes greatly reduced. There is, however, no evidence that follicle-stimulating hormone is directly inhibited by luteinizing hormone. More probably, the effect is due to the more rapid action of the luteinizing hormone on the cells of the membrana granulosa, so that the follicle at an early stage in its development becomes luteinized and, subsequently, is unable to grow normally. Since ovulation does not occur under these conditions the corpora lutea of ovulation, which are responsible for much of the weight of the stimulated ovary, are absent in ovaries of rats stimulated with pituitary extracts containing an excess of luteinizing hormone.

The role of the luteinizing hormone in the determination of the maximum response of the ovaries of intact rats has recently been shown by Deanesly [1939]. Simultaneous injection of an extract containing luteinizing hormone only (chorionic gonadotrophin) decreases the response obtainable with extracts possessing a high proportion of follicle-stimulating hormone (mare serum gonadotrophin). On the other hand, an increase in the response might be expected if these two preparations were injected consecutively into the hypophysectomized rat. From experiments now in progress, this seems to be true.

It is very probable that a proper balance of the two hormones, secreted in correct sequence, is necessary for the induction of normal ovarian activity. The only extracts yet encountered that will effect a true replacement of ovarian function in the hypophysectomized rat are those

prepared from the anterior pituitary gland of the horse. Recent experiments on anoestrous ferrets promise a similar result.

SUMMARY

1. The gonadotrophic properties of extracts of pregnant mares' serum and of horse pituitary gland have been compared by a study of their action in hypophysectomized and intact rats and in oestrous rabbits.

2. Extracts from pregnant mares' serum are equally effective when administered in one single dose or in five divided doses; those from horse pituitary gland are ineffective when given in one injection.

3. Other differences were observed which might be attributed to variations in the relative amounts of the two hormones (follicle-stimulating hormone and luteinizing hormone).

(a) The maximum ovarian weight produced in the intact rat by extracts of pregnant mares' serum was twice as great as that found with extract of horse pituitary gland. In hypophysectomized rats, however, the response to the pituitary extracts was slightly greater than that to the serum preparations.

(b) Horse pituitary extracts caused follicle stimulation followed by luteinization; mare serum extracts caused predominantly follicle stimulation with only very slight luteinization of the granulosa cells of the follicles.

(c) Horse pituitary extracts caused ovulation in intact and hypophysectomized rats; mare serum preparations caused ovulation much less frequently in intact rats, and in only one instance in hypophysectomized rats.

(d) Horse pituitary extracts caused ovulation in oestrous rabbits more readily than did extracts of pregnant mares' serum.

4. We conclude that the proportion of luteinizing hormone in pregnant mares' serum is considerably less than in horse pituitary gland in which the mixture of the two hormones is considered to be optimal for ovarian growth in the hypophysectomized rat.

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THE ANTAGONISTIC EFFECT OF POWDERED AND ALCOHOLIC EXTRACTS OF PLACENTA ON THYROXINE IN AXOLOTLS

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It was suggested by Brandt in 1936 that the placenta exerts an inhibitory effect on thyroxine circulating in the maternal and foetal blood. Experiments are now reported confirming this suggestion.

In feeding experiments with amphibia it has been found [Brandt, 1935] that thyroxine inhibits growth and increases the rate of metamorphosis, whilst placenta has a precisely opposite action, namely, that it increases growth but delays metamorphosis.

Axolotls are so sensitive to thyroxine that even small doses of that substance will induce in these animals changes (alteration of body proportions, shrinking of gills and loss of weight) which can be accurately determined and which may, therefore, be used as a measure of the effect of the thyroxine. For comparative experiments of this kind one of the authors [Brandt, 1933] has already pointed out that it is necessary to use 'animals of equivalent constitution', i.e. of the same stock and of the same generation. The reactions of such siblings lend themselves to comparison extraordinarily well, and it is felt that data obtained from even a small number of animals of equivalent constitution kept under constant conditions of water, temperature, artificial aeration, illumination and diet are much more valuable than that from a large number of animals differing in stock, age, size and conditions of rearing.

To demonstrate the presence in the placenta of a substance inhibiting the action of thyroxine a number of axolotls were fed on beef plus thyroxine, and to some of them was given, in addition, either placenta or placental extract. In those animals which received placenta or placental extract, in addition to thyroxine, metamorphosis was delayed or prevented and growth continued.

EXPERIMENTAL METHODS

Axolotls of the same spawn, selected and maintained according to the criteria set out above, were used in our experiments. Each animal was kept in a glass tank containing 4 litres of tap-water. To the water of each aquarium was added 1 ml. of a solution made by dissolving 10 mg. of

thyroxine in a mixture of 10 ml. of water and 0.4 ml. of 0.1 N caustic soda. With each feed each animal received approximately the same amount of beef. In the course of 36 days the animals were fed 15 times. Animals C and D received only the basic ration of beef. Animal B was given, in addition, 40 mg. of powdered dried placenta which had been prepared by the method of Brandt (British Patent No. 490643, 25th Nov. 1937). Animals A & E had their basic ration plus 40 mg. of the alcohol soluble fraction of placenta. To prepare this fraction, some of the powdered dried placenta was extracted with ethyl alcohol at room temperature—the extraction being continued with frequent agitation for a week. The mixture was filtered and the filtrate was evaporated under reduced pressure at a temperature not exceeding 40° C. and resulted in a gummy residue.

The following measurements of the axolotls were made:—total length, length and width of head, length and width of trunk, distance between fore and hind limbs on the same side, length of tail, height of tail crest, length of right forearm, width of right forearm, length of middle right gill, body weight—as already described [Brandt, 1936].

The animals were measured at the beginning of the experiments and again when one animal showed the first signs of metamorphosis. These data are set out in the Table.

EXPERIMENTAL RESULTS

Table I indicates the results of the measurements at the beginning of the experiment and after three weeks.

The weight of animal A increased from 37 to 40 grammes, that of animal E from 45 to 50 grammes and that of animal B from 39.5 to 41 grammes. The weight of the animals C and D decreased from 49 to 37 grammes and 37 to 23 grammes, respectively. Animals A and E showed increases in length of body, length of trunk, width of trunk, distance apart of the limbs, length of the tail and length of limbs. A comparison of the length of the gills and the height of the crest shows that in B the gills preserved their original length and that the height of the crest decreased by only 0.5 mm. In animals A and E the reduction of the length of the gills was 0.1 and 0.6 mm. respectively, and that of the crest 1.0 and 0.9 mm. respectively. On the other hand, in animal D the gills had disappeared completely at the 21st day, whilst in animal C the gills were reduced to small remnants at their bases at the 34th day after the start of the experiments.

CONCLUSIONS

The experiments clearly indicate that the placenta contains some substance which inhibits the effect of thyroxine on axolotls, and, further, that this fraction can be extracted with alcohol and yet remain active.

Supplement to beef and thyroxine	Axolotl A			Axolotl B			Axolotl C			Axolotl D			Axolotl E		
	Alcohol-soluble fraction of placenta			Powdered dried placenta			Nono			Nono			Alcohol-soluble fraction of placenta		
	On	On	Change	On	On	Change	On	On	Change	On	On	Change	On	On	Change
Measurement	29.4.40	6.6.40	(cm. or g.)	29.4.40	6.6.40	(cm. or g.)	29.4.40	6.6.40	(cm. or g.)	29.4.40	6.6.40	(cm. or g.)	29.4.40	6.6.40	(cm. or g.)
Total length . . .	16.1	17.5	+1.4	16.4	16.6	+0.2	17.2	16.6	-0.6	16.6	15.8	-0.8	17.5	18.4	+0.9
Length of head . . .	3.2	3.2	0.0	3.3	3.3	0.0	3.3	3.0	-0.3	3.1	2.6	-0.5	3.2	3.2	0.0
Width of head . . .	2.8	2.8	0.0	2.8	2.8	0.0	2.8	2.7	-0.1	2.7	2.2	-0.5	2.8	2.8	0.0
Length of trunk . . .	7.8	8.0	+0.2	7.8	8.0	+0.2	7.7	7.6	-0.1	7.8	7.5	-0.3	7.6	8.2	+0.6
Width of trunk . . .	2.2	2.5	+0.3	2.2	2.5	+0.3	2.5	2.3	-0.2	2.1	1.9	-0.2	2.3	2.4	+0.1
Distance between fore and hind limbs on same side of body . . .	4.4	4.8	+0.4	4.0	4.4	+0.4	4.5	4.5	0.0	4.3	4.3	0.0	4.0	4.1	+0.1
Length of tail . . .	9.3	9.5	+0.2	8.6	8.6	0.0	9.5	9.0	-0.5	8.8	8.3	-0.5	9.9	10.2	+0.3
Height of tail erect . . .	3.0	2.0	-1.0	3.5	3.0	-0.5	3.5	1.5	-2.0	3.2	1.2	-2.0	3.2	2.3	-0.9
Length of right forearm . . .	2.1	2.2	+0.1	2.2	2.3	+0.1	2.2	2.1	-0.1	2.0	2.0	0.0	2.0	2.1	+0.1
Width of right forearm . . .	0.4	0.4	0.0	0.3	0.3	0.0	0.4	0.4	0.0	0.4	0.3	-0.1	0.4	0.4	0.0
Length of middle right gill . . .	1.3	1.2	-0.1	1.5	1.5	0.0	2.2	0.4	-1.8	2.1	0.0	-2.1	1.8	1.2	-0.6
Weight . . .	37.0	40.0	+3.0	39.5	41.0	+1.5	49.0	37.0	-12.0	37.0	23.0	-14.0	45.0	50.0	+5.0

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THE ASSAY OF OESTRONE IN THE GUINEA-PIG

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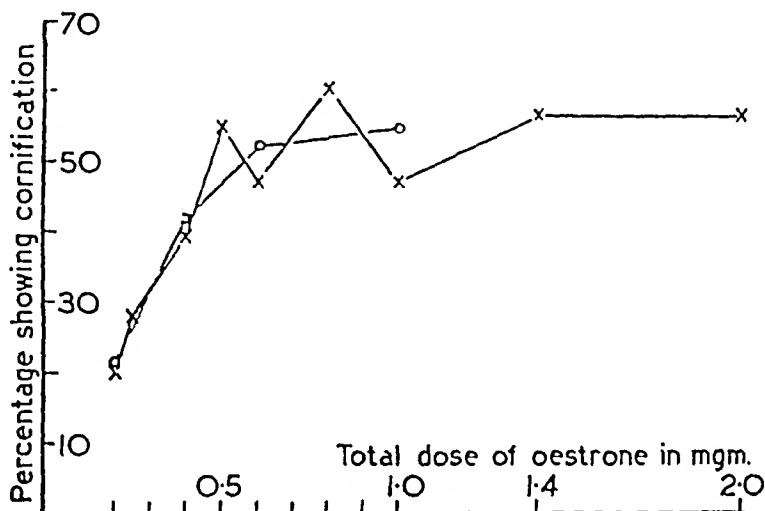
It is now fully appreciated that very great species differences exist in the realm of reproductive physiology. Hain & Robson [1936], for instance, have shown that there is a greater difference between the rat and the mouse units of oestrone, however administered, than can be accounted for on the simple basis of difference of weight. They investigated in great detail the effect of oestrone in oil given in four equal doses over thirty-six hours. The present communication describes the results of giving oestrone in this way to the guinea-pig.

METHOD AND RESULTS

Smooth haired guinea-pigs obtained from several dealers were used. They were ovariectomized at about 250 g. A stock of about twenty animals was maintained during the period of the experiment, about three years, by adding new animals as required to replace casualties. The animals gained in weight during the experiment. No injections were given until a month after the removal of the ovaries, or until a month had elapsed since a previous course of injections. The oestrone (British Drug Houses, Ltd., M.P. not lower than 255° C.) was dissolved in alcohol and added to the necessary volume of sesame oil, the alcohol then being blown off; it was injected subcutaneously in four equal doses on the mornings and evenings of two successive days. Vaginal smears were made by means of a loop from the fourth to the ninth day after the first injection; they were taken at intervals of approximately four hours from 8 a.m. to 10 p.m. (since black-out conditions, the last smear has been made at 5 p.m.). The smears were fixed in a mixture of alcohol and ether and stained with methylene blue and eosin; in this way the cornified cells are stained a clear pink whereas all the other cells are blue. In agreement with Hain & Robson a smear was considered positive or fully cornified when it contained more than 95% of fully cornified pink staining cells. The presence of leucocytes, but not of red cells, made a smear negative. Each point on the graph represents the percentage of a group of forty animals giving a positive reaction.

At first a solution containing 0.05 mg. of oestrone per ml. of sesame oil was used; the results are indicated by the circles on the graph. It can be seen that on increasing the total dose from 0.6 to 1 mg. the number of

animals showing full cornification did not increase. It was then supposed that the relatively large volume of oil might be exercising some inhibitory or toxic effect. Accordingly a more concentrated solution containing 0.25 mg. of oestrone per ml. was tried; the results in this case are indicated by crosses on the graph. This curve practically coincides with the first; the differences are much less than the standard error, which is about 11%



Graph showing the relation between the total dose of oestrone in oil administered to spayed guinea-pigs and the percentage of animals showing full vaginal cornification. Each O or X represents a group of 40 animals.

O—O each ml. of oil contained 0.05 mg. of oestrone.

X—X each ml. of oil contained 0.25 mg. of oestrone.

for 40 observations at 50%. In spite of increasing the dosage up to 2 mg. no further increase in the incidence of full cornification was obtained. The smallest dose producing a positive result in 50% of the animals was about 0.5 mg. in both series. The time of occurrence of full cornification was very variable (from the fifth to the ninth day), but the greatest number of fully cornified smears was obtained on the eighth day after injections began. Initial experiments, in which smearing commenced as soon as the vagina opened, showed that fully cornified smears never occurred on the third day, and only very exceptionally on the fourth day, after injections began.

DISCUSSION

Because of the shape of the curve relating dosage to response, and because of the variability of the time of response, the guinea-pig cannot be considered a suitable animal for the assay of oestrone. Under exactly the same experimental conditions and using the same criteria, Hain & Robson

found that the dose producing cornification in 50% of animals (unit) was 0.0033 mg. for the rat and 0.00009 mg. for the mouse. The guinea-pig unit, 0.5 mg., is thus about 150 times the rat unit and about 5,500 times the mouse unit. The guinea-pigs weighed from 400 to 600 g. and were thus only two or three times the weight of the rats used by Hain & Robson. The discrepancy between these species cannot, therefore, be explained simply on the basis of difference of body-weight.

Dempsey, Hertz & Young [1936] showed that sexual receptivity could not be produced at all regularly in the spayed guinea-pig with oestrin only; they say that their results are 'difficult to reconcile with the view that oestrin alone is responsible for the production of heat'. Oestrus, defined as sexual receptivity, has been produced much more certainly by giving oestrin followed by progesterone. Unfortunately these workers give no record of the vaginal smear picture produced by this treatment—but it would almost certainly be dioestrous. Using somewhat larger doses than those used by Dempsey *et al.*, Bell & Robson [1936] found that a pro-oestrous smear produced by oestrin alone was replaced by a dioestrous smear when progesterone was given in addition. But there is no doubt that there is a stage in the normal oestrous cycle of the guinea-pig when a large proportion of the cells in the vaginal smear are cornified [Stockard & Papanicolaou, 1917; Bacsich & Wyburn, 1940]. The last two workers in a personal communication give it as their opinion that the maximum proportion of cornified cells found during a normally occurring oestrus would be about 90% but that many animals would show a smaller number. Further, leucocytes may persist through all stages of the cycle, although this is not common [Young, 1937]. It would appear that our criteria of full cornification—at least 95% cornified cells with no leucocytes—are artificial and exacting; but the fact remains that it is possible with adequate dosage of oestrin to reach this high standard in 100% of rats and mice.

It is difficult to find any explanation for our failure to produce full vaginal cornification in more than about 55% of guinea-pigs. The possibility that the oily vehicle was toxic seems to have been ruled out. The shape of the curve does not suggest that even higher dosage would have been effective; the rat and the mouse curves show no indication whatever of a falling off at 50%. It is unlikely that the anterior pituitary gland could play any part since its hormones do not appear to act directly on the vagina. It may be that the spacing of the injections used by us is not the optimum for the guinea-pig, but the exploration of this question would have taken us away from the original purpose of this work, namely, to make a direct comparison between the guinea-pig and the rat and mouse. If regeneration of ovarian material had occurred [see Parkes,

Fielding & Brambell, 1927] this might account for the falling off after 50%, especially as the higher doses were given at the end of the experiment. It was our practice to remove with the ovary the whole of the uterine tube and a portion of the uterine horn; regeneration in these circumstances is unlikely. Further, animals which did not react to the highest dosage of oestrone were examined histologically for evidence of ovarian regeneration with negative results.

SUMMARY

Spayed guinea-pigs were injected with oestrone in sesame oil (four injections in thirty-six hours). 0.5 mg. oestrone produced full vaginal cornification in 50% of the animals; this is many times the dosage required in rats and mice, when allowance is made for differences in body-weight. Increasing the total dose of oestrone to 2.0 mg. produced very little increase in the number showing full cornification, but no explanation of this result can be offered.

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PRECIPITINS IN THE SERUM OF RABBITS IMMUNIZED AGAINST PURIFIED SERUM GONADOTROPHIN

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HORMONES of known chemical structure have been artificially linked to proteins to form antigens in which the hormone acts as specific haptene. Immunization with such an artificial hormone antigen will result in the production of antibodies capable not only of forming a precipitate with the complete antigen or with the hormone haptene *in vitro* but also of inhibiting the characteristic physiological action of the whole antigen or the haptene when this is administered parenterally to test animals [Clutton, Harington & Yuill, 1938].

In spite of numerous attempts, however, it has hitherto not been possible to establish the antibody nature of the antihormones evoked by the prolonged administration of natural hormone extracts. The majority of investigators have used crude extracts of the anterior lobe of the hypophysis as immunizing antigens. The use of such crude extracts has invariably yielded sera which, in addition to antihormones, contain antibodies specific for antigens characteristic of the species rather than the hormone. Frequently reliance has been placed on absorption of species-specific antibodies by contact with the serum of the animal from which the immunizing extract was obtained. Such absorption, however, does not necessarily remove all species-specific antibody, and it is possible, on the other hand, that protein which is a constituent of normal serum may be acting as a carrier of the hormone in an antigenic complex.

In order to avoid as far as possible the difficulties of interpretation which may arise from the presence of multiple antibodies evoked by such complex extracts, it is necessary to use for immunization only the most highly purified extracts available. Of these purified extracts the most readily available are gonadotrophins from the urine of pregnant women or from the serum of pregnant mares.

The precipitins in antisera prepared by the immunization of rabbits against highly purified urinary gonadotrophins have already been studied [van den Ende, 1939*a*, *b*]. The conclusion was reached that the hormone preparation used, in spite of its high degree of concentration as indicated by its activity per unit weight, was still antigenically complex, and there-

fore not suitable for quantitative immunological investigations. It was decided, therefore, to undertake a similar investigation with an extract of the serum of pregnant mares. The extract chosen for the production of antisera was so highly purified as to contain approximately 1200 I.U. of gonadotrophin per mg. and was free of chemically demonstrable protein.

Suhman [1939] has shown that the serum of rabbits, immunized by daily subcutaneous injection of purified serum gonadotrophin for a period longer than 3 months, was actively antigonadotrophic and contained complement-fixing antibodies. These antibodies he showed to be of two types, of which one was species-specific, reacting with horse serum but not with proteins of animal species other than the horse, while the other was specific for gonadotrophic hormone, irrespective of its species of origin. The latter antibody, however, differed from the former in its extremely rapid deterioration on storage, and its irregular presence in the serum of immunized animals.

Precipitins in the serum of monkeys immunized against purified serum gonadotrophin have been demonstrated by Gustus, Meyer & Dingle [1935], but the precipitating action of the sera was weak, and the appearance of precipitins did not coincide with the appearance of antigonadotrophic activity. Meyer & Wolfe [1939], on the other hand, found that immunization of monkeys with purified gonadotrophin did not result in the production of precipitins, whereas whole serum from pregnant mares evoked precipitins as well as antihormones. In the latter case, however, the increase and decrease of precipitins and antihormones with repeated injection of serum from pregnant mares coincided.

The experiments here described were undertaken to determine whether the antibodies evoked by serum gonadotrophin, and demonstrable by the precipitin reaction or by the anaphylactic reaction *in vitro*, are related to the so-called antihormones. In view of the numerous recent observations suggesting that mare serum gonadotrophin is, or is associated with, a glycoprotein [Goss & Cole, 1940; Li, Evans & Wonder, 1940], its immunological relationship to scroglycoid [Hewitt], a glyco-protein of normal horse serum, was also studied. Interest attaches also to an investigation of the specificity of the demonstrable antibodies, in the light of the demonstration by Rowlands [1938] that the antihormone evoked by highly purified mare serum gonadotrophin inhibits the physiological action of this extract, while other gonadotrophins, including that present in extracts of horse pituitaries, are unaffected.

METHODS

Biological assay of extracts

These were kindly performed by Dr. I. W. Rowlands. The biological activity of the extracts used in this investigation was determined by their

ability to stimulate the ovaries of the immature rat. Groups of 5 or 10 rats weighing 40–50 g. were injected subcutaneously once daily for 5 days with the extract dissolved in a standard volume of 1.0 ml. of distilled water. The rats were killed 24 hours after the last injection, the ovaries and uteri dissected, and, after fixation in Bouin's fluid overnight, weighed from 70% alcohol.

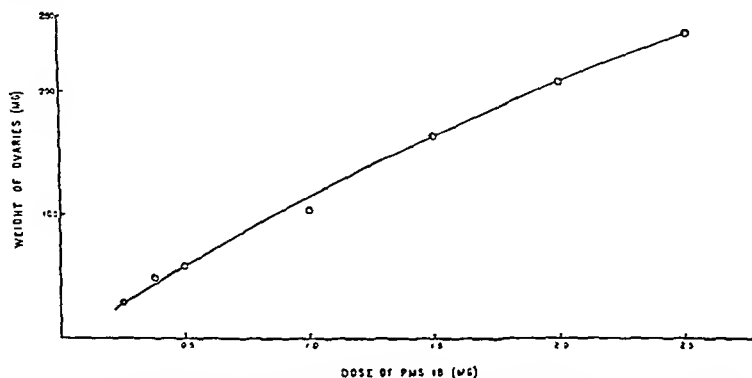


FIG. 1. Curve showing the average weights of ovaries in groups of 5–10 rats given varying amounts of PMS 18 (Pregnant mares' serum gonadotrophin).

A dose-response curve (Fig. 1) was constructed for the extract (PMS 18) which was used in the biological tests for the antigonadotrophic power of the antisera. From this curve the comparative activity of all other extracts was determined in terms of international units.

Biological activity of antisera

The activity of each serum sample was determined by its ability to inhibit the action on the ovaries of the immature rat, of a standard amount (0.375 mg.) of the extract PMS 18. Extract and antiserum were injected simultaneously on opposite sides of the animals once daily for 5 days, subsequent treatment being the same as that described for the assay of the extract. A group of 5 or 10 rats was used for each antiserum.

The degree of inhibition which is observed can be calculated by determining from the curve (Fig. 1) the amount of the extract corresponding to the response obtained. Subtraction of the amount so calculated from the quantity of the extract injected gives the amount of hormone inhibited by a known amount of antiserum. All the results are expressed as the amount of extract (PMS 18) inhibited by 1.0 ml. of the antiserum under test.

Preparation of inhibitory sera

Each of 8 rabbits was given daily subcutaneous injections of 0.1 mg. of a highly purified gonadotrophic extract from the serum of pregnant mares (PMS 22). This extract was selected because of its high degree of purity,

as judged by its activity per unit weight, and because of its almost complete freedom from serum proteins. The animals were bled after $3\frac{1}{2}$ months of such immunization, and thereafter at monthly intervals. Injections of hormones were suspended for 2 or 3 days preceding each bleeding.

After separation, any of the sera that were cloudy from precipitation of lipoids were cleared by passage through a porcelain filter. Each serum sample was tested by the 'ring test' method for the presence of precipitins for PMS 18. From the sera obtained from all the rabbits at any one date of bleeding, a pool was made to which each serum contributed an equal volume. In this way 10 representative pools of serum were obtained corresponding to the 10 dates of bleeding (A to J). All antisera were stored in the frozen state.

Precipitin reactions

As a qualitative test for the presence of precipitins, ring tests were done; increasing dilutions of antigen in 0.1 ml. volume were layered over an equal volume of undiluted antiserum and the tubes examined at $\frac{1}{2}$, 1 and 2 hour intervals for the presence of rings.

For the quantitative estimate of precipitins the optimal proportions method of Dean & Webb [1926] was employed. The delicacy of the precipitates and slowness of the reaction made it necessary to use a relatively high concentration of antiserum in the tests (1 : 2.5). Rough and fine tests were performed according to the method described by Taylor, Adair & Adair [1932] for the estimation of proteins by the precipitin reaction. The amount of antigen at optimal proportions with 1.0 ml. of each antiserum was calculated from the results of the fine tests.

All the antigens used were tested also with normal rabbit serum. No significant non-specific precipitation was found to occur.

RESULTS

Precipitin reactions

In a previous paper the results of an investigation on the precipitins in antisera prepared against urinary gonadotrophins were described [van den Ende, 1939a]. Multiple zones in the optimal proportions test and the presence of free antigens, even in the zone of gross antibody excess, led to the conclusion that the purest preparations of the urinary gonadotrophins were still antigenically complex. The present investigation has shown that the more highly purified gonadotrophic extracts from the serum of pregnant mares are less complex, and give a single zone of precipitation in tests with homologous antiserum. The presence of free antigen and antibody could moreover be shown only in the tubes immediately adjacent to that in which the two reagents are present in optimal precipitating

proportions. The extract, therefore, consists predominantly of a single precipitating antigen. Even the most highly purified preparation available (PMS 22) contains, however, traces of at least one other antigen. This is shown by the results of absorption experiments, in which contact with normal horse serum was found to remove precipitins for this antigen completely, while removing only part of the precipitins for the homologous gonadotrophin.

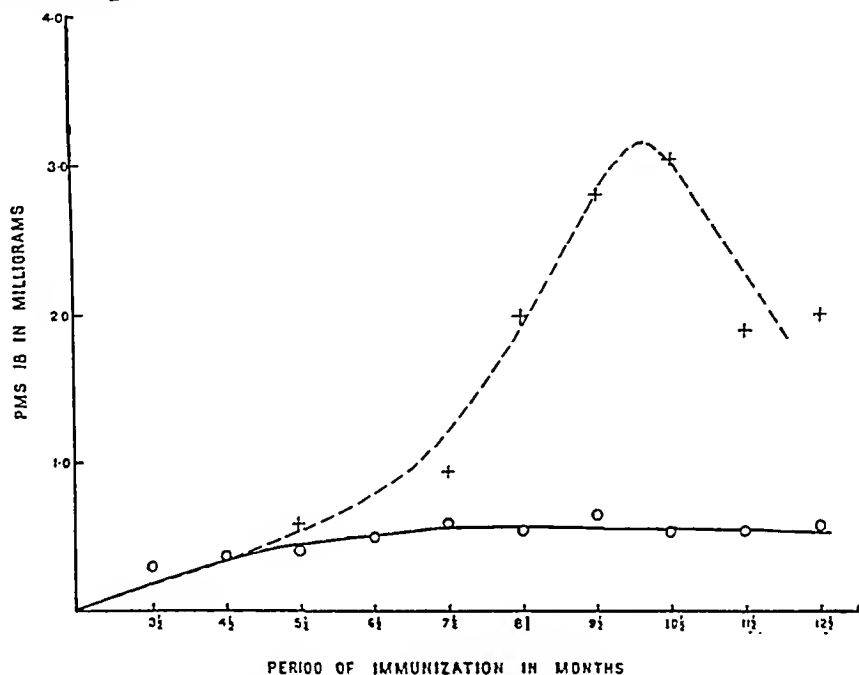


FIG. 2. O ——— O shows the amount in milligrams of PMS 18 at optimal proportions in the precipitation reaction with 1.0 ml. of antiserum; x — — — x the amount of PMS 18 inhibited by 1.0 ml. in the rat test.

Comparison between precipitin content and antihormone activity

Because of the large amount of antigen required for these tests it was necessary to use as test antigen a purified gonadotrophic extract of pregnant mare serum (PMS 18), other than that used for the immunization of rabbits—PMS 22, of which only a small amount was available.

The results are expressed graphically in Fig. 2. The figure shows that no correlation exists between the precipitin content of the sera and their biological inhibitory power. Precipitins are seen to increase up to the 5th bleeding (E), after which they remained approximately constant for the remaining period of the experiment. Biological inhibitory power, on the other hand, increases more rapidly until at the 8th bleeding (10th month

of immunization), when the amount of hormone inhibited in biological tests is almost six times as much as the amount at optimal precipitating proportions with the same amount of antiserum. The biological inhibitory power was diminished in the antisera obtained at the 9th and 10th bleedings (I and J).

The power of the antisera to precipitate gonadotrophic extracts from serum of pregnant mares, whole horse serum and extracts of normal horse serum was also determined. The amounts of each of these antigens at optimal precipitating proportions with 1.0 ml. of antisera C-J were determined by the Dean & Webb method.

Table I

Antiserum bleeding

Antigen	C	D	E	F	G	H	I	J	
PMS 18	0.42 33.6	0.5 40.0	0.6 48.0	0.55 44.0	0.65 52.0	0.55 44.0	0.55 44.0	0.6 48.0	mg. units
PMS 22	not tested	0.068 87.0	0.075 96.0	0.068 87.0	0.086 110.0	0.08 102.0	0.075 96.0	0.07 90.0	mg. units
NMS	± 3.5 nil	4.1 nil	5.0 nil	5.5 nil	6.3 nil	6.0 nil	5.0 nil	6.0 nil	mg. units
SG 75	1.5 nil	1.8 nil	2.0 nil	1.7 nil	2.2 nil	2.0 nil	1.7 nil	2.2 nil	mg. units
Normal HS	0.11 nil	0.16 nil	0.14 nil	0.17 nil	0.19 nil	0.20 nil	0.19 nil	0.21 nil	ml. units
Pregnant MS	not tested	not tested	not tested	0.16 25.6	0.20 32.0	0.20 32.0	not tested	0.20 32.0	ml. units

Table I shows the content of antisera in precipitins for purified gonadotrophic extracts from serum of pregnant mares (PMS 18 and PMS 22); an extract from the serum of a non-pregnant mare (NMS); the fraction of normal horse serum precipitated between 66.6 and 75% saturation with ammonium sulphate, containing a high proportion of seroglycoid (SG 75); normal horse serum (Normal HS); and the serum from a pregnant mare (Pregnant MS). The results are expressed as the amount of each antigen at optimal precipitating proportions with 1.0 ml. of antiserum as well as the equivalent hormone activity of these values in international units. Particular interest attaches to the positive results in optimal proportions tests obtained with that fraction of normal horse serum precipitated by 75% saturation with $(\text{NH}_4)_2\text{SO}_4$ containing a high proportion of a carbohydrate-rich protein, seroglycoid [see Hewitt, 1937; Rimington & van den Ende, 1940]. The main antigen components of horse serum, crystalbumin and globulin, have been found to give no precipitates with the antisera in

optimal proportions tests. In ring tests, using undiluted antiserum, only slight precipitation occurred with globulin when present in concentrations of more than 1 mg. per ml.; and no precipitation could be obtained with albumin.

The results show further that precipitins for all the antigens tested increase at the same steady rate during the first months of immunization, reaching a maximum at about the 6th month after which they remain more or less constant. There is, moreover, no relation between the amounts of the different antigens at optimal proportions with 1.0 ml. of any one antiserum and the hormone content of these antigens as determined by biological assays. It will be seen, for example, that 1 ml. of the antiserum from bleeding H is at the optimal precipitating proportion with 44 units of PMS 18, 102 units of PMS 22 and 32 units of Pregnant MS.

Rowlands [1938] has shown that antiserum prepared against gonadotrophin from the serum of pregnant mares was capable of inhibiting only the hormone from that source, but no other gonadotrophin whether from the pituitary gland or pregnancy urine of the same species (horse) or a different species. It would be of interest to determine whether or not similar specificity was shown by the precipitins.

Specificity of the precipitins

Sulman [1939] from his investigations on complement-fixing antibodies in antigonadotrophic sera concludes that hormone-specific antibodies, as well as antibodies for species-antigens other than the hormone, were present in fresh antiserum. He claims, however, that the hormone-specific antibodies disappear rapidly on storage. In the present investigation precipitation (ring) tests were performed on several of the antiserum samples within the first 3 days after bleeding, and again after several months of storage at -10° C. The results of tests with gonadotrophic extracts from the pituitary glands of the horse, ox and man; purified extracts from the serum of pregnant mares PMS 18 and 22; an extract from normal mares serum (NMS) prepared by a method identical to that used in the preparation of PMS 18; an extract from the urine of pregnant women, UP 27, as well as whole serum and individual serum proteins, are recorded in Table II. They show no significant alteration of precipitin titres by such prolonged storage. The results show that precipitation occurs with horse, as well as human pituitary and urinary gonadotrophins. There is in addition, however, precipitation with serum antigens of these species, and as the pituitary and urinary extracts cannot be regarded as free from serum proteins no significance attaches to these results. Gonadotrophic extracts of ox pituitary do not react with the antisera against pregnant mare serum gonadotrophins.

Table II

Antigen	Fresh	After 4 months' storage
PMS 22 (5 mg./ml.)	1/3125	1/3125
PMS 18 (10 mg./ml.)	1/625	1/625
NMS (10 mg./ml.)	1/125	1/125
Horse pituitary (10 mg./ml.)	1/125	1/125
Horse serum	1/6250	1/31,000
Human pituitary (10 mg./ml.)	Zone	Zone
	1/25-1/625	1/25-1/625
Human serum	Zone	Zone
	1/3125-1/75,000	1/1250-1/150,000
UP 27 (10 mg./ml.)	1/125	1/125
Ox pituitary (10 mg./ml.)	nil	nil
Horse albumin (5 mg./ml.)	nil	nil
Horse globulin (5 mg./ml.)	?1/5	?1/5
Horse seroglycid (5 mg./ml.)	1/125	1/625

It appeared significant that well-marked precipitation occurred with the seroglycid fraction of normal horse serum, whereas no precipitation occurred with the albumin which had been purified by repeated crystallization and which contained no carbohydrate; and only slight precipitation occurred with globulin. Like seroglycid the gonadotrophic preparations from pregnant mare serum are highly soluble and rich in bound carbohydrate. It became important, therefore, to determine whether the gonadotrophin in serum existed in the form of a complex in which seroglycid acted as the antigenic carrier, or whether seroglycid occurred in purified gonadotrophic preparations simply as an impurity, concentrated with the hormone by the methods used for its purification.

Absorption experiments

To show the effect of removing antibodies, and especially of the precipitins for seroglycid, several antisera were absorbed at optimal proportions with normal horse serum, seroglycid, or both, for 2 hours at 37° C. and 24-48 hours in the ice box. Such absorption results in no significant reduction of the biological inhibitory power of the antisera, in spite of the fact that absorption by either horse serum or seroglycid diminished the precipitating power of the antisera for serum gonadotrophin, and absorption with both horse serum and seroglycid resulted in the complete removal of precipitins, even for the gonadotrophic extract against which the antisera were prepared.

It is apparent from these results that although seroglycid was present in the gonadotrophic extract used for the production of the antisera, and although precipitins for seroglycid were consequently present, these precipitins were not responsible for the antigonadotrophic action of the antisera. It has been found, moreover, that antisera prepared by immunizing rabbits against a fraction of normal horse serum, rich in sero-

glycoid, possessed no inhibitory action on serum gonadotrophin. It has further been found that antisera prepared against whole normal horse serum, or against its albumin or its globulin fraction, did not inhibit the action of PMS 18 on the ovaries of immature rats.

*The demonstration of antibodies in the antisera by anaphylaxis
in vitro*

Attempts to use the uterine plain muscle from guinea-pigs actively sensitized with the purified gonadotrophins failed, because of the extreme degree of spontaneous activity shown by uteri of guinea-pigs treated with serum gonadotrophins.

Virgin female guinea-pigs weighing 225–250 g. were, therefore, passively sensitized by the intraperitoneal injection of 1.0–2.5 ml. of antiserum. Forty-eight hours after injection the guinea-pigs were stunned by a blow on the head and exsanguinated. The dissected uterine strips were suspended in Loeke's solution in a bath of 15 ml. volume, according to the method described by Dale [1913].

Results. The purified gonadotrophic extracts PMS 18 and PMS 22, in doses of 0.5–5.0 mg., regularly gave rise to typical maximal anaphylactic contraction of the sensitized uteri. Normal horse serum in a dose of 0.1 ml. also elicited a typical contraction of the sensitized uterine muscle, but after desensitization to that dose it was still capable of maximal response to PMS 22, the antigen used for the preparation of the sensitizing antiserum. A uterus similarly desensitized to horse serum was, however, incapable of responding to PMS 18, given in amounts capable of eliciting maximal contraction of a uterus similarly sensitized and not previously treated with horse serum (see Fig. 3). If, however, desensitization to horse serum was rendered more complete by repeated contact with large doses, an appreciable reduction of the sensitivity to PMS 22 is also produced. The result of a typical experiment is shown in Fig. 4. Fig. 4 shows further that this reduction of sensitivity to PMS 22 is no greater if, instead of normal horse serum, the serum of a pregnant mare (gonadotrophin content 160 I.U./ml.) is used for the desensitization. This result supports the evidence obtained with the precipitation reaction that the antibodies of the precipitin type present in the antigonadotrophic sera and acting here as specific sensitizer of the plain muscle are directed against constituents of horse serum, other than the gonadotrophic hormone.

The results of precipitation reactions have shown that even the most highly purified preparations of the serum gonadotrophin contain traces of ordinary serum antigens as impurities. One of these probably makes up the bulk of the impurities, and has been identified as seroglycoid. It has been possible to confirm these findings by the anaphylactic reaction of

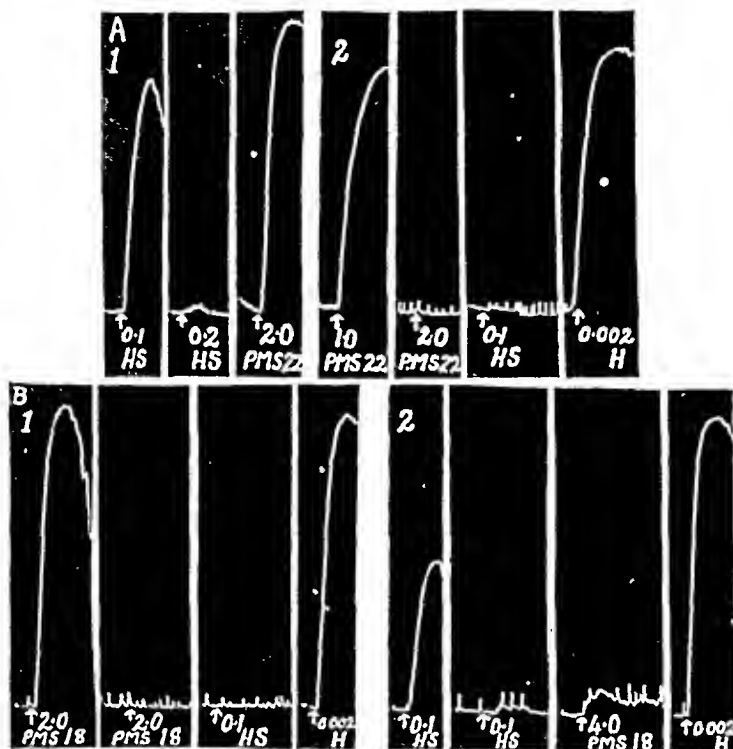


FIG. 3. Response of the uterine horns (1 and 2) of two guinea-pigs (A and B) passively sensitized with antiserum to PMS 22 (purified gonadotrophin from the serum of pregnant mares). 0.1 HS: 0.1 ml. normal horse serum; 0.002 H: 0.002 mg. histamine.

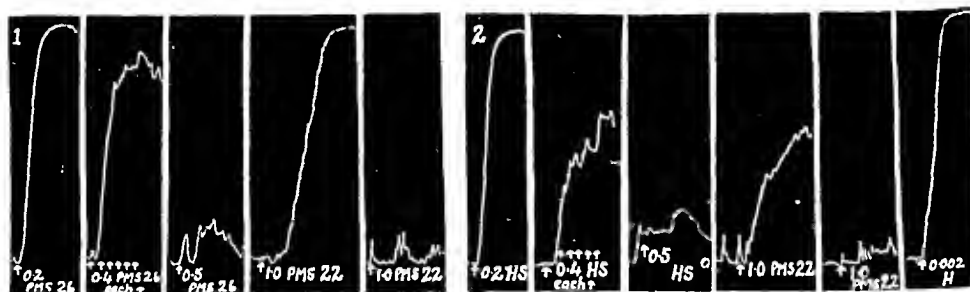


FIG. 4. Response of two uterine horns (1 and 2) of a guinea-pig passively sensitized with antiserum to PMS 22. 0.2 PMS 26: 0.2 ml. of whole serum from a pregnant mare (gonadotrophin content 160 I.U. per ml.).

plain muscle. Thus Fig. 5 shows the response obtained with a uterus passively sensitized with an antiserum against normal horse serum. Fig. 7 shows the result of a typical experiment in which the uterine muscle, passively sensitized with the 'antigonadotrophic' serum responds with a small, transient and gradual contraction to horse serum globulin, with no reaction to horse serum albumin, and, subsequently, with a typical, almost maximal response to normal horse serum. A fraction of normal horse serum rich in seroglycoid, on the other hand, is capable of evoking a typical response even when applied in smaller test doses than globulin or albumin (see Fig. 8). Fig. 8 also shows, however, that practically complete desensitization to this seroglycoid does not desensitize the plain muscle to the homologous antigen (PMS 22), and, further, that similar desensitization to an extract prepared from the serum of a non-pregnant mare (NMS), according to the method employed in the manufacture of PMS 18, fails likewise to desensitize to the homologous antigen. That the antigenic difference between NMS and PMS 22 cannot be dependent on the difference in hormone activity between these extracts is shown, however, in Fig. 6. This figure illustrates the results obtained with the uterine plain muscle of a guinea-pig passively sensitized to PMS 22, and shows that a single typical response of one uterine strip to 0.5 mg. PMS 22 results in desensitization to this extract as well as to PMS 18 (which is prepared by a method identical with that used in the preparation of NMS) in a dose of 10 mg. The second horn of the same uterus, after a single response to 5.0 mg. of PMS 18, is incapable of reacting to contact with the same extract in an amount (30 mg.) which contains 2400 I.U. of gonadotrophin. It still gave a typical though incomplete anaphylactic reaction, however, to 1.0 mg. PMS 22, which is equivalent to only 1200 I.U. of gonadotrophin.

It seems probable that the difference between PMS 22, on the one hand, and NMS and PMS 18 on the other, is dependent on the different methods employed in the preparation of these extracts, and that in the case of PMS 22 the method involved the precipitation of seroglycoid together with the gonadotrophin. It has previously been shown that a single contact with a small dose of horse serum will desensitize a passively sensitized uterus to PMS 18 as well as to horse serum, but not to PMS 22. If, for the passive sensitization, an antiserum is used which has been absorbed at optimal precipitating proportions with seroglycoid, then sensitivity to PMS 22 and horse serum is induced, but a single response of plain muscle thus sensitized to horse serum results in desensitization to the homologous antigen (PMS 22) as well as to horse serum (see Fig. 9). Moreover, the successive single applications of horse serum and seroglycoid in small doses to a uterus passively sensitized with unabsorbed serum resulted in



FIG. 5.

FIG. 5. Response of the uterus of a guinea-pig passively sensitized with an antiserum to normal horse serum.

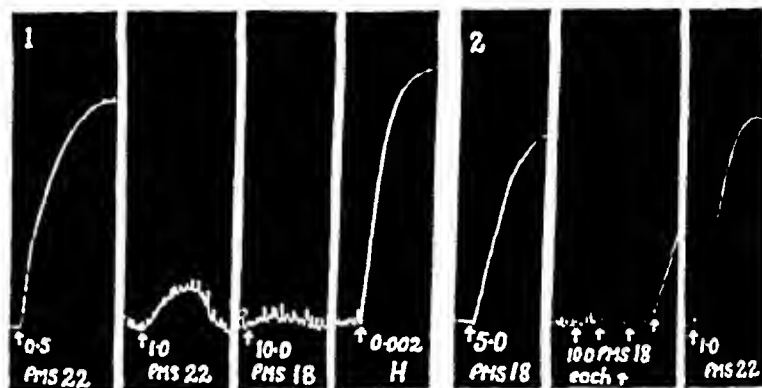


FIG. 6.

FIG. 6. Response of two uterine horns (1 and 2) of a guinea-pig passively sensitized with an antiserum to PMS 22.

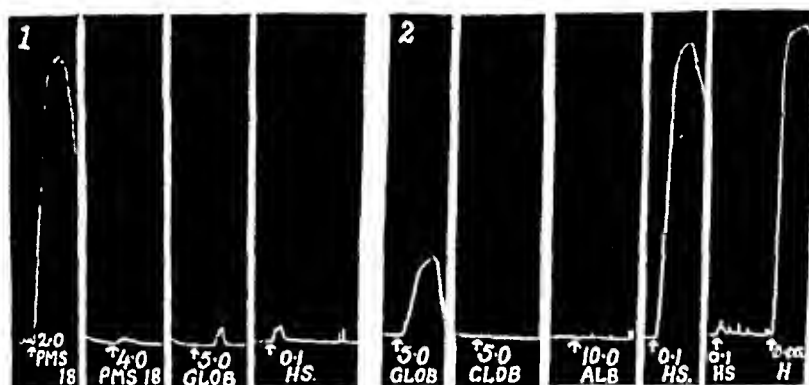


FIG. 7. Response of two uterine horns (1 and 2) of a guinea-pig passively sensitized with antiserum to PMS 22. 5.0 GLOB: 5.0 mg. normal horse serum globulin; 10.0 ALB: 10.0 mg. normal horse serum-albumin.

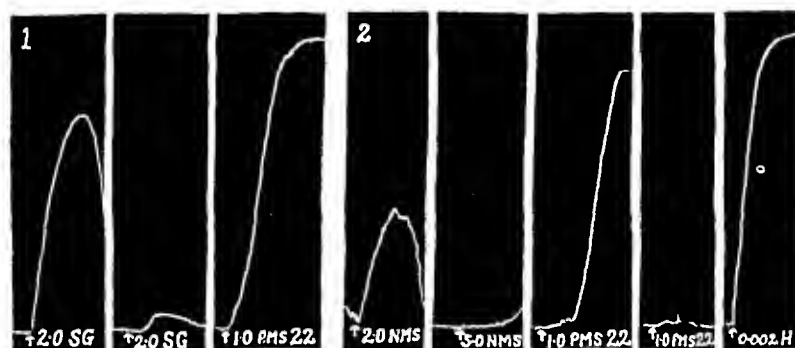


FIG. 8. Response of two uterine horns (1 and 2) of a guinea-pig passively sensitized with an antiserum to PMS 22. 2.0 SG: 2mg. horse seroglycoid; 2.0 NMS: 2.0 mg. of an extract prepared from the serum of non-pregnant maros by a method identical to that employed in the manufacture of PMS 18.

typical anaphylactic responses to both antigens, leaving the plain muscle then incapable of responding to further contact with either of those antigens, or with PMS 22 in the doses employed in this investigation.

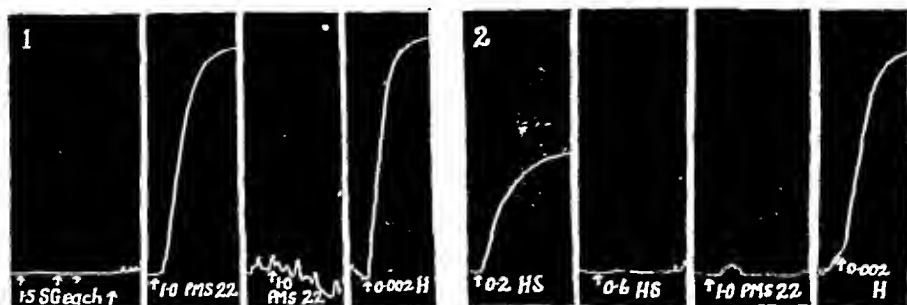


FIG. 9. Response of two uterine horns (1 and 2) of a guinea-pig passively sensitized with an antiserum to PMS 22 from which precipitins for seroglycoid had been removed by adsorption. 1.5 SG: 1.5 mg. horse seroglycoid; 0.2 HS: 0.2 ml. whole normal horse serum.

DISCUSSION

The experiments here reported have shown the presence of precipitins in antisera from rabbits immunized against the most highly purified preparations of serum gonadotrophin available. The precipitins were present in such amounts that their measurement by Dean & Webb's optimal proportions method was possible. The antisera were capable also of inducing specific sensitivity of the plain muscle of guinea-pigs.

Although the gonadotrophic extract used in the preparation of the antisera contained no chemically demonstrable protein, these antisera invariably contained precipitins for normal horse serum. The extract reacted, moreover, though feebly, with antisera prepared by immunizing rabbits against normal horse serum.

The initial experiments showed that, after removal of precipitins for normal horse serum, by its addition to the antiserum in optimal precipitating amount, the antisera still contained precipitins which reacted *in vitro* with the gonadotrophic extract used for their preparation. It was thought possible, therefore, that these residual precipitins might be hormone-specific. Further experiments showed, however, that even the precipitins which remain, after absorption with the optimal precipitating amount of whole horse serum, are not hormone-specific, but can be specifically absorbed by an antigen which is a normal constituent of horse serum, but which constitutes only a small fraction of the total serum proteins. It has been possible to identify this antigen as seroglycoid [Hewitt].

Immunization against the gonadotrophic extract has resulted, therefore,

in the production of at least two distinct precipitins, one of which is specific for seroglycoid and is present in larger amounts than the second, which reacts with a different antigenic component of horse serum. Absorption, therefore, with the optimal amount of untreated horse serum, which contains only a very small proportion of seroglycoid, did not appreciably reduce the precipitins for the latter antigen.

The demonstration in these antigonadotrophic sera of antibodies to seroglycoid, suggested several interesting possibilities. For instance, seroglycoid may be acting as a protein carrier for the hormone, which would account both for the presence of antibodies to seroglycoid in the antigonadotrophic sera, and for the high carbohydrate content which observers have attributed to purified gonadotrophic extracts. The results of these investigations have shown, however, that this is not the case. Another purified and highly active gonadotrophic extract was shown by anaphylactic methods to be almost entirely free of seroglycoid. Further, the absorption of precipitins for seroglycoid, as well as for the unidentified antigenic component of horse serum, although removing completely the precipitins against serum gonadotrophic extracts, did not significantly reduce the biological inhibitory power of the antisera. An antiserum prepared by the immunization of rabbits against horse seroglycoid possessed no antigonadotrophic action against the horse serum gonadotrophin.

Comparison of the rate of appearance of antigonadotrophic activity in the serum, during the prolonged course of immunization, with the rate of appearance of precipitins for horse serum antigens, shows that precipitins rise to a maximum during the first 6 months of immunization, after which they remain at a constant level, whereas the antigonadotrophic power continues to rise steeply during the first 9 months of immunization and falls during the subsequent 2 months. The antibodies demonstrated by the particular methods employed cannot, therefore, be responsible for the antigonadotrophic action.

The quantities of each of a series of extracts from the sera of pregnant mares or normal horses required to produce optimal precipitation with a fixed amount of antiserum, exhibit a wide range of different gonadotrophic activities. This proves again that the extracts, however much purified, still contain in varying proportions precipitinogens which have no hormone action. Seroglycoid, for instance, although present in the extract (PMS 22) used for producing the antisera, appeared to be absent from another gonadotrophic extract (PMS 18) which was used in the precipitation tests.

Although precipitin tests and the anaphylactic reaction *in vitro* have both failed to demonstrate hormone-specific antibodies, the results afford useful information on the antigenic composition of gonadotrophic extracts from mare serum. The presence of seroglycoid, in the most highly purified

serum gonadotrophins employed, suggests that methods employed for the preparation of seroglycoid may assist in the extraction and purification of the serum gonadotrophin.

The results show also that absorption with whole serum cannot be relied upon to remove completely the species-specific antibodies present, even if for absorption a quantity greatly in excess of the optimal precipitating amount of serum is employed.

SUMMARY

Antigonadotrophic sera prepared by the immunization of rabbits against a highly purified extract from the serum of pregnant mares contain antibodies demonstrable by quantitative and qualitative precipitation reactions, and by the anaphylactic reaction *in vitro*.

The precipitins are directed against antigenic constituents of horse serum not related to the hormone. The most important of these, viz. seroglycoid, is a glycoprotein. This is of special interest in view of the suggestions that the gonadotrophin of pregnant mares' serum is, like seroglycoid, rich in bound carbohydrate. Precipitin content and antigonadotrophic power showed no constant relationship to each other.

Absorption of precipitins from the antiserum, and by seroglycoid, resulted in the removal of all precipitins for the gonadotrophic extracts, without significantly reducing the antihormone activity.

I wish to express my thanks to Dr. I. W. Rowlands who kindly performed the biological tests; Løvens Kemiske Fabrik, København for a generous supply of pregnant mare serum gonadotrophin, and an extract from the serum of non-pregnant mares; the Laboratoire du Dr. Roussel, Paris, for a gonadotrophic extract of pregnant mares' serum; and Burroughs Wellcome & Co., Ltd., for a supply of whole serum from pregnant mares.

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HAIR-LOSS AS DEFICIENCY TEST OF MEDULLECTOMY IN RATS

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THE effects of adrenalectomy on hair-loss have been studied in this laboratory for a period of 2 years, using rats. Experiments reported below are representative of the results now to hand.

METHODS

White and black rats, raised in this laboratory, were used for the experiments. Adult animals, weighing c. 200 g. proved suitable. The hair-loss of young rats was small. Extirpation of the adrenals was effected under ether narcosis by dorsal approach. In sham-adrenalectomy, only one adrenal was actually removed whereas the second gland was merely exposed. In these cases the wounds caused by operation healed much more rapidly than in complete adrenalectomy. In many experiments the rats were rubbed down with a mixture of petroleum and linseed oil (1 : 5) 3 or 4 days before the operation and afterwards maintained in specially disinfected and isolated cages. Moulded hairs were caught on a fine wire mesh, placed under the cages, and weighed. The diet consisted of bread and cereals in abundance and of vegetables. Rubin-Kriek solution was given to drink. The room temperature was about 24° C. The survival time averaged 21 days for males and 25 days for females.

RESULTS

Effect of adrenalectomy on the rate of hair-loss

The time after operation at which hair-loss by adrenalectomized rats began to exceed that of sham-operated rats varied with the sex of the animals. Table I presents the general time averages.

Table I. *Average time after adrenalectomy of onset of enhanced hair-loss*

Sex	No. of experiments	Average time to onset of enhanced hair-loss
Males . . .	57	4.5 days
Females . . .	12	7 "
Castrated males .	2	2-3 "

Table II compares the course of hair-loss by adrenalectomized and sham-operated rats (January-February) during a season of intense hair-moult (cf. Figs. 1-5, Plate I).

Table II

Animal	Sex	Onset time of enhanced hair- loss in days after operation	Hair-loss in milligrams					Duration of augmented hair-loss in days
			Average per day	1st week	2nd week	3rd week	4th week	
Adrenalectomized	M	2	54	176	621	399	—	21
Sham-operated	M	—	8	53	65	—	—	At least 16
Adrenalectomized	F	6	40	137	336	345	348	30
Sham-operated	F	—	7	85	18	—	—	At least 14

Table III compares the figures of hair-loss by normal and adrenalectomized rats in March after the end of the season of hair-moult, the observations in this case being continued for 6 days.

Table III

	Adrenalectomized male	Control male	Adrenalectomized female	Control female
Body-weight in g.	200	200	157	145
Hair-loss in mg.	340	37	275	24

Table IV contains data as to hair-loss by adrenalectomized and sham-operated rats in December. Observations in this series of experiments were continued over 8 days.

Table IV

	Adrenalectomized male	Control male	Adrenalectomized female	Control female
Body-weight in g.	158	157	147	156
Hair-loss in mg.	317	24	184	122

The average maximum figures of daily hair-loss recorded for adrenalectomized rats are set forth below in Table V. Corresponding rates recorded for control rats in a state of quiescence and in a state of active hair exchange are given in brackets.

Table V

Animals	Body-weight	Daily maximum hair-loss
Males . .	200 g.	164 (8, 20) mg.
Females . .	200 g.	90 (15, 35) mg.
Males . .	100 g.	20 (1, 1) mg.

An augmented hair-loss rate prevailed during an average period of 19 days in the case of males and during an average period of 14 days in the case of females (cf. Figs. 1-5, Plate I).

The nature and quality of the hair coat and of the moulted hair

The hair coat of adrenalectomized males loses the wiry character and glossy, metallic lustre which distinguishes the normal adult male; the hair of the adrenalectomized males tends to become softer and more like that found on the female. The hide of female rats remains superficially unchanged after adrenalectomy, but after a certain interval it becomes

evident that adrenalectomized rats, female and male, possess a thinner coat of hair than is usual in normal rats.

Control males shed solitary tough over-coat hairs but not wool. Control females lose their silkier over-coat hairs but may also lose a little wool. During the physiological period of hair-moult both males and females largely shed wool hairs. The effect of operation on males and females is simply to increase the amount of wool shed. In adrenalectomized rats which live for a sufficient length of time, a decline in the rate of wool loss may be observed; in the end the loss of over-coat hairs by adrenalectomized rats equals that of normal rats.

In many cases, bald spots tend to appear in special regions. Skin symptoms ranging in severity from dandruff to large scabbed wound formation were found. These are discussed in greater detail below. As to the case with which the hair could be plucked, no significant difference between adrenalectomized and normal rats was observed.

Hair-loss reactions comparable in speed, intensity and regularity to those evinced in adrenalectomy have not been encountered in any other condition of endocrine shortage or excess. Examinations on this point carried out so far include the following: thyroidectomized, thyrotoxic, castrated and sex-hormone treated rats. Cases of avitaminosis examined for hair-loss reactions include lack of vitamin B complex, vitamin A or vitamin D. In no case were clear-cut hair-loss reactions, such as characterized adrenalectomized rats, encountered. The hair-loss reactions described above must, therefore, be regarded as specific for adrenalectomized animals.

Attempts to inhibit the hair-loss

Observations on the influencing of hair-loss are best carried out when this process is either in its initial stage or at its height. In the phase when the rate of loss is declining, modification of the hair-loss course is difficult or impossible.

Several common adrenal preparations ('Cortin', 'Eschatin', desoxycorticosteron acetate) failed to influence the course of hair-loss. The hair-loss reaction is not, therefore, due to disturbances in the mineral balance, carbohydrate metabolism or circulatory system of adrenalectomized animals, since all these disturbances are adjusted by treatment with the above-named preparations. Also androsterone, testosterone, progesterone, oestrone and prolan were without effect on hair-loss. Pregnancy did not exert any clear influence. Vitamins A, B-complex and E were likewise without effect.

Feeding rations supplemented with rat adrenals, or with extract of rat adrenals prepared at pH 7.3 and with or without addition of 5 mg. cystine, did not modify the course of hair-loss.

Active hair-loss inhibiting preparations were obtained by extraction of fresh cattle adrenals with 0.2N HCl or with alcohol. The active principle was highly sensitive to oxygen and to alkali. Cortin from fresh preparations was ineffective. Either, therefore, cortex contains a second active agent or this agent is located in the medulla of the adrenal gland. When cortex and medulla were separated as completely as possible, by far the predominant quantity of active principle was found in the medulla. The low activity noted for the cortex can probably be completely accounted for as due to the incomplete separation of cortex from medulla. In 6 experiments with 200 g. rats, 0.2–2.0 mg. of medulla still proved active, whereas 25–50 mg. of cortex were necessary to give a significant detectable effect.

Experiments with medullectomized rats

From the results reported above it is to be expected that rats deprived of medulla but possessed of cortex will evince the same hair-loss reactions as are shown by adrenalectomized rats.

Medullectomy was carried out in part according to the method of Evans [1935/36], but generally according to a modification of this method, wherein one adrenal gland was completely removed, and the second adrenal was treated as indicated by Evans. Out of 25 operations carried out, only 4 animals were lost through cortical deficiency. All operations were carried out on males weighing c. 200 g. The operated animals showed largely the same course of hair-loss as did the adrenalectomized rats. The rate of loss exceeded normal about 6 days after the operation (4–8 days). Hair-loss by 200 g. rats continued at an abnormal rate for an average duration of 23 days, the maximum and minimum duration being 9 and 40 days respectively.

The hair-loss of medullectomized rats rose steeply to a maximum within 3–7 days of the operation, remained at the maximum for about a week, then fell sharply, though frequently still exceeded the normal maximum (20 mg.) for considerable time periods after the decline had begun (cf. Figs. 1–5, Plate I). The average maximum loss per 24 hr. for medullectomized rats was 155 mg. (60–250 mg.). The course of hair-loss is exemplified by the following protocol (Table VI) of an experiment with a male medullectomized rat weighing 222 g. Figures in brackets represent the hair-loss rate at the indicated times.

Table VI

Onset of enhanced hair-loss	Maximum	Period of maxi- mum hair-loss	Onset of decline	Duration of decline to normal
5th day (61 mg.)	10th day (191 mg.)	10th–21st day (Av. 96 mg.)	21st day (to 60 mg.)	21st–25th day (60–18 mg.)

After the rate of hair-loss had reverted to normal, a recurrence of the abnormal loss was in no case observed. Visible differences in the quality of the hair coat were only noted in the second week after operation. At this time the hair on the top, nape and shoulders is so thinned as to render the pinkish skin of the albinos just visible. The skin in the neighbourhood of the operation scar is generally completely exposed by this time. Here, as well as on the hair-covered back skin, but particularly at the tail root, yellow flaky patches are frequently encountered.

In the third week after the operation the skin of most medullectomized rats is either bald or thinly covered on the forehead, shoulders or back (cf. Figs. 6-7, Plate I). The skin is thickened, and almost always dandruffed or mealy. Simultaneous baldness at all of the places mentioned is rare. In certain cases the hair coats remain normal or practically normal in appearance despite a marked loss of hair.

Out of 17 medullectomized rats maintained under observation during prolonged time intervals, 13 developed distinct bald spots.

Towards the end of the second week after the operation, firmly attached, pin-head sized, conical, scabbed pimples appear at the forehead, near the upper lip, under the chin, on the nape and on the external sides of the extremities (cf. Figs. 6-7, Plate I). In severe cases, which, however, represent only a small fraction of the total number of animals examined, the entire skin became covered with partly confluent wounds. In no case, however, were the abdominal region or the inner sides of the extremities affected by any of the symptoms described. In a number of cases ulceration reactions failed to develop during the full time the animals were kept under observation. The skin wounds, though frequently of rather ugly appearance, healed rapidly and often had disappeared completely in the 5th week after the operation. By this time, too, renewed hair-growth makes good progress and bald spots are generally covered over with new hair. Adrenalectomized rats show similar symptoms.

At rare occasions old and feeble rats of the laboratory colony spontaneously develop hair and skin symptoms such as have been described above. Medullectomized rats, again, at the crisis of their skin disease, give an impression of senescence.

The regular reversion of the rate of hair-loss to normal and the disappearance of the remaining skin symptoms within several weeks after the operation is readily explained by the hypertrophy of extramedullary chromaffin in the animal organism.

The effect of adrenalin on hair-loss

It has been pointed out that acid extract of adrenal medulla inhibits the hair-loss reaction of adrenalectomized rats. Several experiments showing

the effect of injections of medulla extract in doses corresponding to 0.5–15.0 mg. of tissue are summarized in Table VII.

Table VII

No. of expt.	Operative treatment	Hair-loss in mg.			Relative hair-loss
		Before injection of medulla extract	After injection of medulla extract		
			1st day	2nd day	
23	Medullectomized	89	48	111	1 : 0.54 : 1.3
20	Adrenalectomized	107	55	102	1 : 0.51 : 0.95

In a subsequent series of experiments the effect of pure adrenalin rather than medulla extract was examined. Adrenalin was found to be markedly active. *l*-Adrenalin of Poulenc Frères was used. Adrenalin was injected subcutaneously at 5 p.m. and hair-loss on the following two days was determined. The limiting minimal dose was found to be 2.5–5 μ g. per 100 g. body-weight. A series of such experiments using medullectomized and adrenalectomized rats and medium doses of adrenalin have been summarized in Table VIII. The last column of the table shows the relative rate of hair-loss before, during, and after adrenalin treatment where the former is expressed as 1.0.

Table VIII

No. of expt.	Operative treatment	Adrenalin dose in μ g. per 100 g. body-weight	Hair-loss in mg.			Relative hair-loss
			Before adrenalin treatment	After injection of adrenalin		
				1st day	2nd day	
23	Medullectomized	5-75	99	48	92	1 : 0.48 : 0.92
18	Adrenalectomized	20-100	100	43	142	1 : 0.43 : 1.42

Adrenalin treatment in suitable dosage regularly led to a reduction in the hair-loss rate, but generally failed to reduce the rate to normal even when powerful doses were administered. The best effect was often observed with adrenalin in a weak dose administered either early in the morning or late in the evening. On repeated daily administration of adrenalin a rapid loss in effectiveness, whose cause could not be determined, was observed. When injections were made on alternate days, on the other hand, the action of adrenalin on hair-loss could be uniformly repeated. Vitamin C and cysteine failed to enhance the effect of adrenalin. Addition of cocaine, which is a powerful stimulant of the action of adrenalin on the blood-vessels and smooth muscle system, failed to influence the effect of adrenalin upon hair-loss. In several cases brief incubation of adrenalin at pH 6.5 for 10–15 min. produced an increase of the adrenalin effect; incubation at higher pH for time intervals of more than 8–20 min. completely nullifies the potency of the solution. Inactivation may be effected also by enzymic

means—through oxidative deamination of the side chain as described by Blaschko, Richter & Schlossmann [1937] or by the action of potato phenolase [Blaschko & Schlossmann, 1940].

Attempts to influence hair-loss or its reaction to adrenalin by means of vagal stimulants of the choline group were uniformly unsuccessful. In adrenalectomized, but not in normal rats, ergotamine (0.25 mg.) occasionally evoked violent itch reactions and an increase of hair-loss. As might be expected, also, ergotamine occasionally neutralizes the inhibitory effect of adrenalin on hair-loss.

The question whether the principle regulating hair-loss in adrenal medulla is identical with adrenalin was examined in the following manner: The minimum effective dose of fresh medulla extract was determined. Concurrently the adrenalin content of the extract was assayed by two methods: that of Folin, Cannon & Denis [1913] and that of von Euler [1933]. The average minimum effective dose of extract found in six experiments corresponded to 0.7 mg. medulla. The adrenalin content of the latter was found to be $5\mu\text{g}$. Calculated to 100 g. body-weight of rat (200 g. rats were used) the minimum dose of extract corresponded to 0.35 mg. medulla = $2.5\mu\text{g}$. adrenalin. With synthetic *l*-adrenalin the minimum effective dose was found to be $2.5\text{--}5\mu\text{g}$. The close agreement observed in the respective minimum dose levels appears, therefore, to justify the conclusion that adrenalin or some closely related compound is the hair-loss regulating principle in adrenal medulla.

Influence on hair-loss of substances related to adrenalin

Different substances related to adrenalin, which were available in the laboratory, were tested for their action on hair-loss. The results of these tests are summarized in Table IX.

Table IX

Substance	Dose	Effect
Catechol	5 and 10 mg.	negative
Tyrosine	6 mg.	negative
Diiodotyrosine	7, 5, and 10 mg.	negative
Dioxyphenylalanine	4, 7.5 and 11.5 mg.	negative
Tyramine	5, 10 and 15 mg.	positive
"	1 and 2 mg.	negative
Ephedrine	3 and 10 mg.	negative
Benzedrine	2.5 and 5 mg (<i>per os</i>)	negative

Among the substances tested, tyramine alone proved effective, though even this only in concentrations considerably greater than were necessary in the case of adrenalin. It is pertinent to this result to mention the *in vitro* synthesis of adrenalin from tyramine demonstrated by Schuler and

his colleagues [Schuler & Weidemann, 1935; Schuler, Bernhardt & Reindel, 1936] and Devine [1940]. The formulation of general rules relating chemical constitution with physiological activity in respect of hair-loss must await the extension of the tests to include a greater number of compounds. The inactivity of benzedrine and ephedrine, the low activity of tyramine, and the failure of cocaine to enhance the inhibition of hair-loss by adrenalin support the conclusion that hair-loss inhibition by adrenalin probably has nothing in common with the well-known other effects of this hormone, but should be regarded rather as a further special activity involving a special mechanism.

DISCUSSION

The bulk of the experimental evidence on the function of the adrenal medulla seems to indicate that this portion of the gland plays no significant role in bodily economy during periods of rest. In conditions of emotion, violent muscular exercise, cold, &c., epinephrine, on the other hand, is liberated reflexly. The functional importance of the adreno-sympathetic mechanism in mediating these important physiological responses during emergencies is well established. Studies on animals in which the adrenal medulla had been destroyed by methods which do not limit the functional activities of the adrenal cortex and in which the rest of the sympathetic system has been left intact, however, have given little convincing evidence that animals are less capable of survival following destruction of the adrenal medulla than a normal animal in a condition of stress which involves vigorous muscular activity [De Campos, Cannon, Lundin & Walker, 1929; Harris & Ingle, 1940; Rogoff & Nixon, 1940]. The function of the adrenal medulla is at present more enigmatic than ever. The phenomenon of hair-loss described constitutes a clear case of a deficiency which is caused by removal of the adrenal medulla, influenced by the administration of medulla extract or adrenalin, and is spontaneously corrected after several weeks, probably through the hypertrophy of additional chromaffin tissue. To all appearance no connexion between the hair-loss reaction and other effects of adrenalin has yet been found. The former phenomenon can be measured very soon after the removal of the adrenal medulla; the quantitative assay of the effect is easy and provides a convenient and pronounced indicator of the effect of adrenal medulla or adrenalin. We are aware that the hair-loss and skin reactions may be only a few of the many changes induced by medullectomy, which, taken together, produce the general appearance of senescence. The hair coat appears to form a tissue which is unusually susceptible and rapidly reactive to adrenalin deficiency. Other effects of medullectomy are probably dampened to a greater or lesser extent by the action of extramedullary chromaffin tissue, and all

deficiency effects are probably cancelled within a relatively brief time by the hypertrophy of the latter.

Since the initiation of studies on nutritional deficiency in rats, numerous characteristic skin and hair-coat symptoms have been observed and described. These symptoms have been observed in advanced stages of almost all known avitaminoses, but are not an integral part of the syndromes described. In the case of certain avitaminoses of rats, the skin symptoms serve as a most important indicator of diet deficiency. This is particularly true of the deficiency syndrome which characterizes avitaminosis of the B₂ group. A comprehensive summary of these symptoms has been published recently [Chick, Maerac & Worden, 1940]. The peculiar phenomenon of the greying of the hair of black rats deprived of a vitamin, recently shown to be distinct from the B group, deserves special mention in this connexion [cf. for instance Morgan, Cook & Davison, 1938]. The skin symptoms observed after medullectomy are very different from those found in avitaminoses, and cannot, *prima facie*, be connected with the latter. It may prove significant, however, that severe changes of the adrenals have been found in certain avitaminoses and it should be of interest to examine the effects, if any, of adrenalin upon the skin symptoms observed in those latter [Daft & Sebrell, 1939].

A search of the literature for previous work on the hair and skin symptoms of medullectomized animals disclosed two pertinent references. In the compendium of Biedl [1922], Pende in 1913 is stated to have observed hair-loss after adrenalectomy. Bayer [1929] cites a similar finding by Ferreira de Mira using dogs.

Butcher & Richards [1939], working with rats, have shown that adrenalectomy strongly stimulates the growth of the second hair coat, previously inactive hair buds being found to show signs of development 40 hours after the operation. According to these authors, the effect is dependent on the removal of the adrenal cortex. It appears justified, therefore, in view of this and of our own findings, to conclude that hair-moult in rats is regulated by the adrenal glands.

SUMMARY

1. Adrenalectomy causes a marked, though transient, increase in the rate of hair-loss in rats. The increased loss is largely of wool hairs. Skin symptoms, ranging in severity from dandruff to extensive ulcer formation, are frequent concomitants of the hair-loss reaction.

2. Medullectomized rats show a similar transient increased hair-loss and similar skin symptoms.

3. Acid extract of adrenal medulla can inhibit the hair-loss reaction.



FIG. 6. Medullectomized male, 17th day after operation. Baldness—scabs near the upper lip and on the shoulder.



FIG. 7. Medullectomized male, 21st day after operation. Bald skin on the top, nape and shoulders.



FIG. 2. Medullectomized male.



FIG. 4. Sham-operated male, quiescent stage; collected within 2 days.



FIG. 5. Sham-operated male, hair-moult stage.

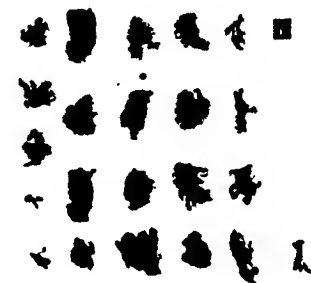


FIG. 1. Adrenalectomized male.



FIG. 3. Normal male, quiescent stage.

The minimum effective dose of extract for a 200 g. rat corresponds to 0.7 mg. of medulla and contains 5 μ g. of adrenalin.

4. Corresponding quantities of synthetic *l*-adrenalin were equally effective in inhibiting the hair-loss reaction.

5. The phenomenon described provides a convenient test of deficiency conditions as yet unknown, which are induced in the organism by medullectomy and is a simple indicator of their modification by medulla extract and adrenalin.

6. No connexion between the effect of adrenalin on hair-loss and other known effects of adrenalin has as yet been found.

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THE EFFECT OF DESOXYCORTICOSTERONE ON THE BODY-WEIGHT OF FROGS

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In a previous experiment [Dow & Zuckerman, 1939], it was found that the body-weight of axolotls tends to fall following the injection either of adrenal cortical extract or of desoxycorticosterone acetate. Since such treatment leads to an increase in body-weight and water retention in adrenalectomized mammals, this finding suggested that, in so far as loss of body-weight indicates a loss of body-water, cortical hormone does not act in the same way in axolotls as in mammals. It was consequently of interest to test its effects in another amphibian species.

METHODS

Animals

Fifty male frogs were used, varying in weight from 8.50 to 28.25 g. Each animal was used for one injection only, and kept (and fed) during, and for 12 hr. before, the experiment in a perforated tin (8×8×4 in.) lined with damp grass and moss. Weighings were made hourly after each injection over a period of 12 hr. and again 24 hr. after the beginning of the experiment. The frogs were weighed in a dry beaker on a swing balance and returned to their tins. The beaker was reweighed immediately.

Injections

Three groups of frogs were used. One control group of ten animals received no injections at all. Each of a second control group of twenty animals received 0.25 ml. of arachis oil. Each of the remaining twenty animals received 0.25 mg. desoxycorticosterone acetate in 0.25 ml. arachis oil. Injections were made with a glass tuberculin syringe subcutaneously in the mid-dorsal region.

RESULTS

Thirteen of the animals given desoxycorticosterone died before the completion of the experiment. The first death occurred 5 hr. after the injection and one or two animals died in every subsequent hour.

The remaining animals survived for at least 30 hr. From 4 hr. after injection the skin of all the animals given desoxycorticosterone became noticeably abnormal to the touch: the usual sliminess was absent and the

skin felt somewhat like damp wash-leather. Moreover, all the animals became inactive about this time and remained so for the whole period they were under observation (30 hr.).

The changes in body-weight are illustrated in the accompanying table, in which the mean body-weight at successive hours after injection is expressed as a percentage of the mean body-weight at the time of injection. The individual figures obtained for any one period show great variation. Consideration of the data indicates that this variation is largely due to the fact that whereas a maintained increase in body-weight occurred in the majority of the surviving animals within 12 hr. after injection, the increase began to show itself at widely differing times.

The data for the animals injected with arachis oil and for the uninjected animals are also collected in the table. No deaths occurred in either group, so that it may be concluded that the 65% mortality in the desoxycorticosterone group was due to the drug itself. There are no significant differences between the uninjected and the oil-injected groups.

Initial weight range (g.) Dose range $\mu\text{g./g.}$	Desoxycorticosterone							
	Uninjected		Arachis oil		All animals		Seven survivors	
	8.50-12.25		10.50-23.50		11.25-28.25		12.50-28.25	
	—		—		8.9-22.2		8.9-20.0	
Hours after injection	No. of		No. of		No. of		No. of	
	obs.	% wt.	obs.	% wt.	obs.	% wt.	obs.	% wt.
1st hr.	10	102.74 \pm 8.9	20	104.17 \pm 0.44	20	99.93 \pm 0.95	7	100.65 \pm 1.33
2nd "	10	102.74 \pm 0.11	20	93.75 \pm 0.51	20	99.04 \pm 0.94	7	100.58 \pm 1.32
3rd "	10	97.06 \pm 0.96	20	100.00 \pm 0.51	20	99.92 \pm 1.59	7	101.31 \pm 1.19
4th "	10	97.96 \pm 0.12	20	100.00 \pm 0.59	20	99.99 \pm 2.40	7	101.17 \pm 2.32
5th "	10	100.00 \pm 0.95	20	97.92 \pm 0.56	19	100.28 \pm 1.29	7	101.38 \pm 2.15
6th "	10	102.74 \pm 0.96	20	102.05 \pm 0.58	19	101.56 \pm 1.36	7	103.33 \pm 2.53
7th "	10	97.06 \pm 0.85	20	100.00 \pm 0.62	18	102.80 \pm 1.37	7	104.32 \pm 2.36
8th "	10	102.74 \pm 0.95	20	100.00 \pm 0.65	16	104.42 \pm 1.98	7	105.23 \pm 3.09
9th "	10	102.74 \pm 0.89	20	97.92 \pm 0.60	12	105.23 \pm 2.17	7	105.80 \pm 3.12
10th "	10	100.00 \pm 0.90	20	97.92 \pm 0.66	11	104.64 \pm 1.86	7	105.47 \pm 1.09
11th "	10	102.74 \pm 1.1	20	95.92 \pm 0.73	9	105.03 \pm 2.18	7	105.78 \pm 2.41
12th "	10	100.00 \pm 0.77	20	95.83 \pm 0.73	9	105.31 \pm 2.47	7	106.35 \pm 2.64
24th "	10	98.82 \pm 0.92	20	95.83 \pm 0.21	7	107.39 \pm 0.44	7	107.20 \pm 0.44

The mean relative body-weight of all the frogs given desoxycorticosterone acetate has been compared by Fisher's 't' test with the corresponding mean weight in the oil-injected animals. The difference became significant ($P = < 0.01$) at 7 hr. after injection, and remained significant up to 24 hr. when weighings were discontinued. The differences are also significant at the 7th hour if the comparison is made between the control group and only those frogs which survived the whole experimental period after the administration of desoxycorticosterone acetate.

The results of this analysis show that desoxycorticosterone leads to an increase in the body-weight of frogs. The irregular incidence of the increase is probably due to variations in relative dosage. Thus mean dosage in $\mu\text{g./g.}$ received by those animals showing a maintained increase beginning in the first 3 hr. is $16.4 \mu\text{g./g.}$, and in those showing a similar effect between 6 and 12 hr., $11.8 \mu\text{g./g.}$

SUMMARY

0.25 mg. of desoxycorticosterone acetate in arachis oil were injected into each of twenty male frogs weighing from 11 to 23 g. Thirteen died within 24 hr. A control group was given oil alone. The mean body-weight of all the hormone-injected animals increased significantly from the 7th hour of the experiment onwards. Frogs thus behave differently from axolotls in their reaction to cortical hormone.

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THE EFFECT OF OESTROGENIC STIMULATION ON THE HUMAN PROSTATE AT BIRTH

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ALTHOUGH the changes which oestrogens induce in the prostate differ in detail from species to species, in general they always begin in epithelial and fibro-muscular proliferation, and usually result in gross enlargement of the organ. This fact is primarily responsible for the belief that the clinical disorder of benign prostatic hypertrophy is also due to oestrogenic stimulation, a view which in large part is responsible for the use of androgens as a counteracting measure in the treatment of the condition. As yet, however, there is little direct information about the effects of oestrogens on the human prostate. For that reason it is of interest to report the observations which form the subject of this paper.

Hamilton, Heslin & Gilbert [1937] state that no prostatic changes were induced in men who were given 13 injections of 50 to 150 μ g. of oestrone over a period of 26 days, their conclusion being based on various indirect clinical signs and on the examination of biopsy specimens removed at the end of the period of injections and at intervals in the succeeding 5 months. Positive results of similar treatment were, however, obtained by Moore & McLellan [1938], who injected 5 men with a total of from 15,000 to 140,000 I.U. of oestradiol benzoate for periods varying between 10 and 31 days. At the end of the period of treatment prostatectomies or transurethral resections were performed, and it was found that marked changes had occurred, particularly in the epithelium of the urethra and the prostatic collecting ducts, which had become deeply stratified, and in places had undergone so-called squamous metaplasia. These changes are similar to those observed in the prostates of new-born infants, and they are regarded by Moore & McLellan as an intensification of those usually observed in the benignly hypertrophied prostates of men not treated with oestrogens.

The peculiar histological characteristics of the prostate at birth, which are detailed below, were first reported by Asehoff in 1894 and by Schlaehta in 1904. In 1904 and 1905 Halban ascribed them, on purely theoretical grounds, to the action of maternal sex-hormones which had passed through

the placenta. These findings and views were given prominence by Burrows [1935], who drew attention to the similarity of the picture of the new-born human prostate with the changes which oestrogenic stimulation produces in mice.

The present paper is concerned both with the histological appearance of the prostate at birth, and with the responses of the organ to injected oestrogen.

MATERIAL

Fifteen infantile prostates were available for study. Two were from babies who had been given, in the course of treatment, 5 mg. of oestradiol benzoate daily for 46 and 50 days respectively. The first died of a congenital cor trilobularis at 7 weeks and 1 day, and the second, at 7½ months, of a severe congenital hydrocephalus.

The remaining thirteen were from infants who had not been given oestrogen and who died at the following ages:

Still-born	5
12 hours	1
1 day	1
4 days (premature)	1
5 days	1
6 weeks	1
8 weeks	1
12 weeks	1
6 months	1

The tissues were removed at times varying between 3 and 72 hours after death. Most were fixed in Formol saline, and a few in Bouin's fluid. Some were cut in the sagittal and others in the transverse plane, most sections being stained with haematoxylin and eosin.

OBSERVATIONS AND RESULTS

The prostate at term and in the first two months of life

Our observations on the normal appearance of the infantile prostate in most places confirm and in some extend the very detailed descriptions given by Aschoff and, in particular, Schlachta. The facts are briefly as follows. At birth the prostate is larger than it is after the third month. The epithelium over the crista urethralis, instead of being of a transitional type, as it is after the third month, is deeply stratified, especially in its central region (Figs. 1 and 2, Plate I). The change rarely extends far above the mouth of the uterus masculinus, nor does it reach to the distal limit of the prostatic urethra. The stratification is due to a process, usually regarded as epithelial metaplasia, which also affects the uterus masculinus and those prostatic ducts which open in the region of the mouth of the

uterus masculinus. In the prostatic ducts the change begins at the 8th month of the foetal life, and in the uterus masculinus, according to Pallin [1901], at the beginning of the 5th.

No 'metaplasia' has been recorded after the 68th day. Schlachta examined sections from 5 infants in the first year of life. Stratified squamous epithelium was found in the prostate of one 68 days old, but none was present in prostates removed from older children. There were 8 specimens in our corresponding control series. Relatively little squamous epithelium was present in the prostate of an infant aged 6 weeks, and none in infants of 8 and 12 weeks. The intensity of the change presumably diminishes progressively after birth.

Schlachta emphasizes the fact that the number of prostatic ducts which show the change varies, but that the change is (a) symmetrical, (b) better marked in the more cephalic than in the more caudal ducts, and (c) also present in isolated urethral glands which sometimes occur in the anterior part of the prostate. The ducts which pass through the pre-spermatic part of the organ ('utricular bed' [Zuckerman, 1938]) to open into the lateral urethral gutters just above the mouth of the uterus masculinus are usually most affected (Fig. 3, Plate I). The change may affect the whole of a duct, or be isolated to only a part, usually, but not always, the region nearest the urethra. The metaplasia begins in one or more foci, generally situated near the urethral opening of the ducts. When the prostate resumes a normal appearance towards the end of the second month of extra-uterine life, it is in these parts of the prostatic ducts that stratified epithelium persists longest. It may be noted that in the first year of life, and until puberty, the prostatic 'glands' consist only of collecting ducts, true secretory alveoli not yet being differentiated (Fig. 4, Plate I). Many of the ducts are not fully canalized, and most show solid lateral and terminal proliferating nodes.

The altered epithelium is made up of one or more layers of very large clear swollen cells, which give a glycogen reaction, and which as a rule overlie one, two or three layers of unchanged small basophil cells (Fig. 5, Plate I). Occasionally, transformed cells appear to be proliferating under a layer of normal cells which become raised from their original position. As the transformation of the originally cuboidal or cylindrical cells (which in normal circumstances line the prostatic ducts and the uterus masculinus) continues, the ducts become much distended, often completely occluded, and sometimes cystic. Often a focus of these cells, arising from a few cells, may block a duct which is otherwise normally lined. Degeneration and desquamation of the stratified cells occurs, and as the desquamated cells disintegrate, many undergo liquefaction and disappear (possibly in part through being discharged into the urethra). Thin strands may be left

passing across the ducts, while small tubular glandular formations, lined by cuboidal cells, sometimes containing 'colloid', not infrequently develop in the thickened walls of the prostatic ducts. They are identical with the similar structures which appear in the prostatic ducts of langur monkeys (*Presbytis entellus*) that have been injected with oestrone [Zuckerman & Sandys, 1939].

The swollen stratified cells, according to Schlachta, sometimes give a mucin reaction, which may also be given by their cell membranes and by the 'degeneration globules' that are found in a mass of swollen stratified cells.

The form of the transformed uterus masculinus varies in shape and size from specimen to specimen. In most it consists of a large distended sac lined with 'metaplastic' squamous epithelium, from the basal and unchanged layers of which irregular cords of cells pass into the surrounding hyperplastic and oedematous stroma (Fig. 6, Plate I). Sometimes these proliferating cords have differentiated to form secondary glands, of the kind observed in the *Entellus* Langur [Zuckerman & Parkes, 1936]. Similar gland-like structures also appear in the wall of the main lumen of the utericle. Occasionally, as in many species of monkey, the utericle is T-shaped in cross-section, its main lumen not being distended (Fig. 7, Plate II). At other times the organ consists of an irregular series of cords of cells passing in all directions from a main lumen (Fig. 8, Plate II), which in one specimen divides to open into the urethra by two mouths.

The epithelium of the ejaculatory ducts is sometimes hyperplastic near the urethra. The epithelium of the seminal vesicles is inactive, and in no specimen was it hyperplastic or metaplastic. On the other hand, the muscular wall of the seminal vesicles is relatively greatly thickened during the latter months of foetal and in the first months of extra-uterine life, a change also suggestive of oestrogenic stimulation [Zuckerman & Sandys, 1939].

The degree to which the susceptible parts of the prostate undergo 'metaplasia' varies according to no apparent rule. The crista may be only slightly and the uterus masculinus greatly changed; sometimes the reverse condition holds. Occasionally the prostatic ducts show little 'metaplasia', and the terminal parts of the ejaculatory ducts are much altered; at other times the reverse condition holds. The available material makes it impossible to decide whether this variation is indicative of variation in the order in which the prostate resumes a normal condition.

The prostate after oestrogenic stimulation

Both the babies who were given oestrogen during treatment died at an age when the prostate would have resumed a normal inactive appearance

(7 weeks and 1 day, and $7\frac{1}{2}$ months respectively). In both, however, the prostate had undergone extensive epithelial changes of a kind associated in other species of primate (and in other mammals) with oestrogenic stimulation, and of the kind normally present in man only just before and just after birth.

The prostate of the infant which died at 7 weeks showed fewer changes than the one which died at $7\frac{1}{2}$ months, although both had received approximately the same amount of oestrogenic hormone. In both the changes were restricted to the urethral epithelium, the uterus masculinus and the uppermost prostatic collecting ducts (Fig. 9, Plate II). The change in the urethra did not extend to the ventral wall and was restricted to the central part of the crista urethralis immediately above and below the opening of the uterus masculinus. In neither were the terminal buds of the ducts in any part of the prostate affected (Fig. 10, Plate II); these parts of the prostatic glandular system appeared identical with the corresponding structures in monkeys that have been injected with oestrogenic hormone. In both specimens, too, the lower part of the prostate was completely unaffected. The metaplastic process (Fig. 11, Plate II) was in large part thus confined to epithelial structures in the utricular bed, which was much enlarged relatively. There was no epithelial change in the ejaculatory ducts, seminal vesicles, vas deferens, or (except in an occasional collecting duct) in Cowper's glands. Cowper's glands were similarly unresponsive to oestrogens in rhesus monkeys [Aykroyd & Zuckerman, 1938].

DISCUSSION

These observations provide direct evidence in support of the hypothesis that the changes which are normally observed in the new-born human prostate are due to the action of oestrogenic hormone which has passed, in the latter half of foetal life, from the maternal organism through the placenta. The response of the new-born human prostate to oestrogen appears to be similar to that of the rhesus monkey in so far as, in many specimens, the human uterus masculinus responds mainly by epithelial stratification [Parkes & Zuckerman, 1935; Zuckerman, 1938]. It is similar to that of the *Entellus Langur* in so far as, in some specimens, the utricle proliferates partly in a glandular manner, and in the restriction of the change to those structures which open into the urethra, and to that part of the urethral epithelium in the immediate vicinity of the mouth of the utricle [Zuckerman & Sandys, 1939]. The response of the new-born human prostate is also similar to that of both species of monkey in so far as the greatest degree of prostatic growth takes place in the stroma

through which pass the uterus masculinus, the upper collecting and the common ejaculatory ducts.

No evidence is yet available to show whether the mature human prostate responds to oestrogenic stimulation in exactly the same way as the infantile prostate. Moore & McLellan [1938] refer not to any changes in the uterus masculinus but only to changes in the urethral epithelium and prostatic collecting ducts, where they are similar to those of the infantile prostate. In view of this observation it would, however, seem unlikely (though not impossible) that the mature utricle would normally proliferate under the influence of oestrogenic stimulation differently from that of the new-born prostate—i.e. that it would undergo a type of glandular hyperplasia which would be indistinguishable from the histological picture seen in the 'middle lobe' of a benignly enlarged prostate, rather than undergo a predominantly vaginal type of response as it does in the new-born infant.

This likelihood does not exclude the possibility that oestrogenic stimulation is in some way concerned in the production of the clinical condition of prostatic enlargement. Both Moore & McLellan, and Burrows, regard the metaplastic changes which are observed in the new-born prostate as being essentially similar to the changes that occur during the process of benign enlargement. Furthermore, there is definite evidence that certain types of enlargement in dogs are due to oestrogenic stimulation [Zuckerman & Groome, 1937; Zuckerman & McKewen, 1938]. To what extent oestrogens are responsible for the human clinical condition can only be determined by further histological study of its earlier stages, and by comparing them with the effects of direct oestrogenic stimulation in adult men.

SUMMARY

1. In the later stages of foetal and in the first two months of post-natal life the human prostate shows changes suggestive of epithelial metaplasia. These changes, which comprise a transformation of small cuboidal or cylindrical cells into a stratified epithelium of large clear cells, are generally restricted to the uterus masculinus, the upper collecting ducts and the epithelium over the summit of the crista urethralis.

2. Similar changes were observed in the prostates of two infants who were given oestradiol benzoate in the course of treatment, and who died (of congenital defects) at an age when the prostate would have resumed a normal inactive appearance.

This fact is taken as evidence in favour of the hypothesis that the changes observed immediately before and after birth are due to the leakage through the placenta of sex-hormone from the maternal organism.

FIG. 1. Sagittal section of prostate of still-born infant. The crista urethralis is deeply stratified. Epithelial metaplasia can be seen in anterior prostatic glands.

FIG. 2. High-power view of crista urethralis of preceding section. $\times 92$.

FIG. 3. Sagittal section of prostate of still-born infant. Distended metaplastic collecting ducts can be seen in the upper part of the organ. The uterus masculinus is the large irregular structure lying between the distended collecting ducts and the true prostatic tissue.

FIG. 4. Prostatic 'glands' in prostate of five-day old infant. The alveoli have not yet differentiated. $\times 92$.

FIG. 5. Collecting ducts in prostate of five-day old infant. The epithelium has undergone metaplasia. $\times 92$.

FIG. 6. Transverse section of prostate of one-day old infant. The uterus masculinus is very small and its epithelium has undergone metaplasia. The epithelium of the crista urethralis has also undergone metaplasia at the level of the section.



FIG. 1.



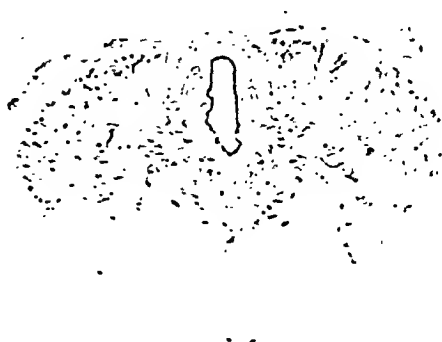
FIG. 2.



FIG. 3.



FIG. 4.



- FIG. 7. Transverse section of uterus masculinus of five-day old infant. The lumen has the T-shape that is observed in rhesus monkeys. Strands of cells from the basal layers of the utricular epithelium are extending into the surrounding stroma. The superficial layers of the epithelium have undergone metaplasia. $\times 92$.
- FIG. 8. Uterus masculinus of eight-week old infant. The organ consists of a diffuse system of proliferating glands extending in all directions. No metaplasia is apparent. $\times 92$.
- FIG. 9. Transverse section of prostate of infant 7½ months old after oestrogen treatment. The utricular bed is very extensive, as is also the uterus masculinus, which has undergone extensive metaplasia. $\times 92$.
- FIG. 10. Prostatic ducts in same specimen showing no effect of the oestrogenic treatment.
- FIG. 11. High power view of metaplastic cells of uterus masculinus of same specimen. $\times 92$.



FIG. 7.



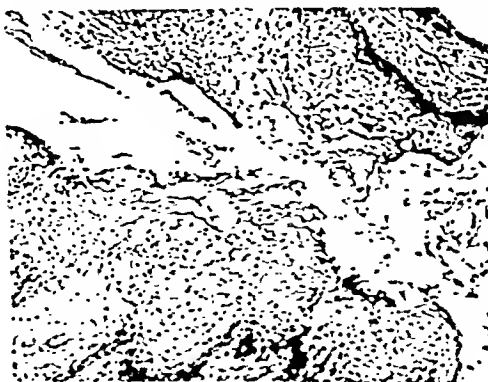
FIG. 8.



FIG. 9.



FIG. 10.



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THE EFFECT OF PREVIOUS ON SUBSEQUENT RESPONSES OF RHESUS MONKEYS TO OESTROGENS

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CONFLICTING statements have recently been made about the view that previous oestrogenic stimulation influences an animal's subsequent responses to oestrogenic hormone. Thus Emmens [1939] writes that there is no correlation in mice 'between the amounts of oestrogen previously received and sensitivity at any given time', except in animals 'which have had one previous injection only or have received no oestrogen for more than six weeks'—such animals being less sensitive than others. In a more recent study devoted to this point alone, Palmer [1941] states the contrary conclusion that a significant positive correlation does actually exist in mice between 'present response and history of response to previous treatment'; the divergence between this and Emmens's finding, Palmer suggests, may be due to the fact that his own observations were made at weekly, and Emmens's at fortnightly intervals, by which time the effect of a dose of oestrogen might have worn off completely. Bishop & McKeown [1941] also show that previous dosage affects subsequent responses in mice. Their results indicate that animals which have been injected for relatively long periods with high doses of oestrogen are less sensitive than animals treated for the same period with low doses.

The data presented below show that the threshold dose of oestrogen, given in a single injection, to which a rhesus monkey will respond varies according to the animal's immediately previous response to oestrogen. By threshold dose is meant the least amount of hormone that is needed to influence the endometrium so that a phase of uterine bleeding follows [Zuckerman, 1937a].

MATERIAL AND METHODS

The observations on which the present paper is based were made in the course of a long-term study, the results of which will be published later, of the relative potency of the following oestrogenic compounds, which were given in a single injection intramuscularly in oil solution:

1. Oestrone
2. Oestrone benzoate
3. Oestradiol
4. Oestradiol benzoate

5. Oestradiol caprylate
6. Oestradiol dipropionate
7. Oestradiol 3-benzoate-17-*n*-butyrate
8. Diethylstilboestrol
9. Diethylstilboestrol dipropionate
10. Hexoestrol
11. Dihydroxy diphenyl hexadiene.

Thirty-one adolescent or mature spayed rhesus monkeys (*M. mulatta*) were used. In testing a compound the first step was to administer a purely arbitrary dose. If this dose was followed after an interval by a phase of uterine bleeding, the dose was reduced and the experiment repeated until a level was reached at which bleeding did not occur. If bleeding did not occur after the first injection, the dose was raised until it did occur. As a general rule the following scale of doses, in mg., was adhered to:

10.0, 7.5, 5.0, 2.7, 2.0, 1.5, 1.0, 0.75, 0.5, 0.4, 0.3, 0.2, 0.1, 0.075, 0.05, 0.025, 0.0125, 0.00625, 0.003125, 0.0015, 0.00075, 0.0005.

In the case of esters the amount given was calculated in terms of free hormone.

After injecting, vaginal lavages were made daily until uterine bleeding occurred or until at least 40 days had passed (60 days in the case of hexoestrol and the hexadiene compound). In no instance was an experiment abandoned (i.e. was it assumed that bleeding would not occur) until the vaginal lavage had assumed the character it has in untreated spayed monkeys. When uterine bleeding occurred, a new injection was given as a rule on the second or third day of bleeding.

Table I. *Latency in days before uterine bleeding occurs after single injections of oestrogens. All values, except the line at the foot of the table, are percentages.*

Days	Oestrone	Oestrone benzoate	Oestradiol	Oestradiol benzoate	Oestradiol dipropionate	Oestradiol caprylate	Oestradiol benz-butyrate	Stilboestrol	Stilboestrol dipropionate	Hexoestrol	Dihydroxy diphenyl hexa-dieno	All cases
1-10	38.2	16.7	27.6	8.7	5.7	—	—	10.3	3.0	12.5	12.5	12.6
11-20	50.1	55.6	48.4	69.6	68.6	70.6	36.4	79.3	80.0	60.4	81.3	62.6
21-30	8.8	16.7	10.4	17.4	17.1	29.4	36.4	6.9	5.0	16.7	—	15.1
31-40	2.0	5.5	6.8	—	5.7	—	21.2	3.5	3.0	4.2	6.2	5.7
41-50	—	5.5	—	4.3	2.9	—	3.0	—	3.0	2.0	—	1.9
51-60	—	—	3.4	—	—	—	3.0	—	—	4.2	—	1.2
61-70	—	—	3.4	—	—	—	—	—	3.0	—	—	0.6
71-80	—	—	—	—	—	—	—	—	3.0	—	—	0.3
No. of cases	34	18	29	23	35	17	33	29	36	48	16	318

Table I shows that in only 4% of 318 experiments in which the injection was followed by uterine bleeding was the latent interval longer than 40 days and in the case of no single substance did this percentage exceed 10.

RESULTS

Before a preliminary idea of the potency of any of the tested substances was obtained, it was usual to alter the doses (in the scale chosen) in a somewhat haphazard way from experiment to experiment. It soon became apparent, in comparing different animals, that the threshold appeared lower in animals which were tested with decreasing effective amounts of hormone than in animals which were tested with increasing amounts, beginning at an ineffective level. As soon as this fact was appreciated, each animal, so far as possible, was tested against each substance in both ways. With practically no exception it was found, as in the comparison between animals, that the threshold dose for any given animal was lower when the doses were decreased, starting at an effective level, than when they were raised from an ineffective level. In either case the threshold did not remain constant over a period of time, although its variation was only within narrow limits. These findings are exemplified by the records detailed in Tables II-V, which are representative of the entire group of some fifty similar series of experiments.

Table II. *Uterine bleeding in a rhesus monkey (70.34-50) after single injections of oestradiol 3-benzoate-17-n-butyrate. Depending on whether the doses were either being decreased or increased, the animal responded or failed to respond to 0.10 mg. and 0.50 mg. respectively. Uterine bleeding had occurred in the experiment immediately preceding the first of this series*

Sequence of experiments	Amount of hormone (mg.)	Result	Threshold (mg.)
1	0.05	No bleeding	
2	0.075	"	
3	0.50	"	0.5-1.0
4	1.00	Bleeding	
5	0.75	"	
6	0.50	"	
7	0.30	"	
8	0.20	"	0.1-0.2
9	0.10	No bleeding	0.1-0.2
10	0.20	Bleeding	
11	0.10	"	0.075-0.1
12	0.075	No bleeding	
13	0.10	"	
14	0.20	"	0.2-0.3
15	0.30	Bleeding	
16	0.20	"	<0.2

Table III. *Uterine bleeding in a rhesus monkey (3.45–51, 63–73) after single injections of diethylstilboestrol dipropionate. Experiments 7 and 8 were separated by an interval in which the animal was injected with oestradiol benzoate (for details see Table IV). Uterine bleeding did not follow the last injection of oestradiol benzoate*

It will be seen that the threshold for diethylstilboestrol dipropionate varied between 0.0125 and >0.075 mg. depending on whether the animal was being injected with decreasing effective amounts or with increasing amounts beginning at an ineffective level.

Sequence of experiments	Amount of hormone (mg.)	Result	Threshold (mg.)
1	0.50	Bleeding	
2	0.40	"	
3	0.30	"	
4	0.10	"	
5	0.05	"	
6	0.025	"	
7	0.0125	No bleeding	0.0125–0.025
8	0.025	"	> 0.025
9	0.30	Bleeding	
10	0.20	"	
11	0.10	"	
12	0.075	"	
13	0.05	"	
14	0.025	"	
15	0.0125	No bleeding	0.0125–0.025
16	0.025	"	
17	0.05	"	
18	0.075	"	> 0.075

Table IV. *Uterine bleeding in a rhesus monkey (3.52–62) after single injections of oestradiol benzoate. Bleeding had not occurred in the experiment (7 of Table III) immediately preceding the first of this series. The threshold proved to be higher (0.75–1.5 mg.) when the doses were gradually increased from an ineffective than when they were decreased from an effective level (0.05–0.075 mg.)*

Sequence of experiments	Amount of hormone (mg.)	Result	Threshold (mg.)
1	0.40	No bleeding	
2	0.75	"	
3	1.5	Bleeding	0.75–1.5
4	1.0	"	
5	0.5	"	
6	0.4	"	
7	0.3	"	
8	0.2	"	
9	0.1	"	
10	0.075	"	
11	0.05	No bleeding	0.05–0.075

Table V. *Uterine bleeding in a rhesus monkey (435.5-21) after single injections of hexoestrol. Bleeding had not occurred in the experiment immediately preceding the first in this series. Variation in the threshold as in Tables II, III, IV*

Sequence of experiments	Amount of hormone (mg.)	Result	Threshold (mg.)
1	0.5	No bleeding	1.0-5.0
2	1.0	"	
3	5.0	Bleeding	
4	2.7	"	0.50-0.75
5	1.0	"	
6	0.75	"	
7	0.5	No bleeding	4.0-10.0
8	0.75	"	
9	4.0	"	
10	10.0	Bleeding	0.75-1.0
11	7.5	"	
12	4.0	"	
13	2.7	"	> 1.0
14	2.0	"	
15	1.0	"	
16	0.75	No bleeding	> 1.0
17	1.0	"	

DISCUSSION

These observations show that a single dose of oestrogen, both above and about the threshold effective to stimulate uterine bleeding, is more likely to produce a response in a spayed rhesus monkey if it immediately follows a phase of uterine bleeding than if it is given after a preceding dose which was insufficient to produce bleeding. Experiment, of which details will be given elsewhere, also shows that, even if the preceding dose is high enough to produce bleeding, a subsequent dose is less likely to do so the longer the interval between its administration and the cessation of the previous bleeding.

Since uterine bleeding following oestrogen-administration is due to a gradual fall to a critical level of the concentration of oestrogen in the body [Zuckerman, 1937*a, b*], these findings would also seem to suggest that the small amount of oestrogen that may be left in the body at the time of bleeding continues to exercise not only an important influence on the uterus for some days, but a greater influence than larger doses which have proved ineffective. An alternative and more likely explanation is that the uterus is much more responsive to oestrogenic stimulation immediately after uterine bleeding begins than when it has returned to the inactive state normal in ovariectomized monkeys—a conclusion which has obvious clinical bearings.

The experiments also show that a statement of the threshold dose of oestrogen (given in a single injection) for an individual monkey can only be a series of values relating to the different circumstances of administration that have been discussed above. Further data are being collected to discover the conditions under which extreme values vary.

Finally, it may be noted that the fact that an ineffective low dose results in the raising of an animal's oestrogen threshold may be an explanation of the difficulties that are encountered in finding the level of daily administration at which recurrent phases of uterine bleeding will occur [Zuckerman, 1941].

SUMMARY

A series of experiments on 31 spayed rhesus monkeys, using 11 different oestrogenic compounds, has shown that the minimum single dose of oestrogen effective in producing uterine bleeding is lower when injections are begun at an effective high level and then decreased, than when the injections are increased from an ineffective low level. They also suggest that the uterus is much more responsive to oestrogenic stimulation immediately after uterine bleeding begins than when it has returned to the inactive state normal in ovariectomized animals.

This finding appears to have clinical bearings.

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PRECURSORS OF OESTROGENS

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A VERY large number of compounds is now known which reproduce or imitate the action of the naturally occurring oestrogens. The outstanding researches of Dodds and his co-workers have shown that, while potencies equalling or approaching those of the natural substances seem to be possessed only by those synthetic compounds which appear to be closely related to them in structural configuration, the structure of other more weakly oestrogenic compounds may diverge considerably from this type.

Stroud [1940] has shown, however, that when diphenyl, diphenyl ether, diphenylmethane, stilbene and γ : δ -diphenyl- β : δ -hexadiene are injected into female rabbits, phenolic metabolic products are excreted, which are usually more active oestrogenically than the parent compounds. It might be supposed, therefore, that the activity shown by the non-phenolic precursors is due to the formation of the phenols *in vivo*. The question thus arises, which of the apparently active substances so far described are direct oestrogens, and which require to undergo a metabolic change in the body before they become oestrogenic?

A simple method of investigating this question was suggested by the observation that some of the synthetic oestrogens exhibit widely different ratios of activity from that of oestrone, with different methods of administration. Thus, α -phenyl-stilboestrol causes vaginal cornification in 50% of mice in a dose of 15 μ g. by injection, having approximately one two-hundredth of the potency of oestrone, which causes a 50% response in an injected dose of 0.075 μ g. When given directly into the vagina, by the technique described below, the dose of the former required to produce cornification is still 10 μ g., whereas oestrone thus given is active in the relatively minute dose of 0.00029 μ g., giving a ratio of over 30,000. The synthetic compound, scarcely if at all more active when given locally than when injected, must differ in its mode of action from oestrone, which exhibits such a greatly enhanced potency when given locally. The simplest explanation is that α -phenyl-stilboestrol is itself inactive, and must be transformed in the body to an active metabolite. If such compounds were absorbed from the vagina into the circulation, as from an injection elsewhere, the metabolite would presumably return to the vagina in no greater dose or concentration than that which would reach it when the same dose of the parent substance was given subcutaneously.

A series of compounds has therefore been investigated with a view to separating true oestrogens from supposed precursors or 'pro-oestrogens', by the method of comparing the doses of each required to cause vaginal cornification in the same proportion of mice when given (a) subcutaneously, and (b) intravaginally, with the expectation that pro-oestrogens would be needed in about the same amount intravaginally as subcutaneously; and that true oestrogens would be effective in a much smaller dose when given into the vagina. This would obviously still leave open the possibility that some substances which are classified as true oestrogens on this basis owe their apparently direct activity to local transformation to active compounds, a relatively small local dose being sufficient when the transformation does not require systemic absorption. Since, however, this may well be true even of some of the natural oestrogenic hormones, if not, indeed, of all, the distinction between true oestrogens and pro-oestrogens, made on the basis of the relation between local and systemic effectiveness, may be regarded as a real one.

TECHNIQUE

Intravaginal assays

It is fairly generally agreed that oil solutions are not very effective when introduced into the rat or mouse vagina, but that aqueous or 50% water-glycerol solutions will enable a very small quantity of the natural oestrogens to take effect [Lyons & Templeton, 1936; Emmens, 1939a; Freud, 1939; Mühlbock, 1940].

Preliminary tests on ovariectomized mice with international standard oestrone dissolved in distilled water, physiological saline or 50% water-glycerol showed that little difference exists between the effectiveness of these media. Since the solubility of various compounds seemed highest in 50% glycerol, and the viscosity of the vehicle aids its retention in the vagina, this solvent was chosen for further tests. These were conducted by giving two intravaginal doses in 0.01 ml., or occasionally 0.02 ml., one on each of two consecutive days, by means of a Trevan micrometer syringe, fitted with a blunt needle having a small aperture near to the rounded end. A volume of 0.01 ml. is the most that can be given with any safety, as leakage from the vagina occurs readily with greater quantities, but it was occasionally necessary to give 0.02 ml. in order to introduce sufficient material, when dealing with weakly active compounds.

Smears were taken at first in the evening of the 2nd day, and then twice daily, in the morning and evening, for two further days. Since practically all positive responses were detected by the 2nd and 3rd smears, the 1st and 5th were sometimes omitted in later tests. As with other assays of this type, only smears with no leucocytes and with

cornified or nucleated epithelial cells were scored as positive, all others as negative.

It was impossible to get the higher doses of many compounds into complete solution, and the material had to be introduced as a finely ground suspension or crystalline mush. Care was taken to ensure, as far as possible, the homogeneity of such preparations, since a series of samples of 0.01 ml., if taken from an uneven suspension of large particles, might vary greatly. It was usually impossible to administer a higher dose than 2-4 mg. of any compound, but in a few cases a paste was successfully made which facilitated the introduction of up to 20 mg. total dose. In only one instance was such a high dose of material manifestly unabsorbed by the time of the first smear, which contained crystals of the material (*p*-tertiary-amyl-phenol). In the great majority of tests, it may be assumed that absorption was complete or very nearly so, as careful search for residual material in smears has been unsuccessful.

Systemic assays

These assays were carried out as already described by Emmens [1939a], two injections being given in oil, one on each of two consecutive days, and smears taken on the 3rd and 4th days. The same criterion of response was used as in the intravaginal series.

Expression of results

The amounts of a substance required to give 50% of positive responses in each test were usually calculated from results with 10-30 mice, after preliminary assays with series of graded doses, one per mouse. Sometimes, lack of material prevented such a detailed investigation; this is indicated by placing 'ca.' before the estimate in the tables. The more detailed investigations with a few compounds are referred to separately below.

The accuracy of the comparison of the systemic and local doses is in most cases not great, and varies from compound to compound. Time-to-time variation in response has not been strictly controlled, although other precautions such as 'priming' at regular intervals have been taken. Attention to the finer points would have been unnecessary, as will be apparent from the tables, since we are dealing with differences of the order of 100:1 as between the oestrogens and pro-oestrogens. The basis of discussion will be the ratio between the systemic and local median effective doses, twice the standard error of which is normally of the order of -50% and +100%, rarely more. Thus, ratios which should actually be unity may fall between 0.5 and 2.0, and those which should actually be 100, between 50 and 200. However, the experimental findings have

generally indicated that the ratios for the true oestrogens are more accurate than those for the pro-oestrogens, often because it was possible to use more animals per substance. Those given for oestrone, oestradiol, oestriol and diethylstilboestrol have standard errors of not greater than $\pm 15\%$.

RESULTS

Dose/response lines for intravaginal assays

In Table I are shown the results of an investigation into the responses to oestrone, oestradiol, oestriol and diethylstilboestrol. The main points

Table I. *Response of ovariectomized mice to oestrogens applied intravaginally in 50% water-glycerol, by two doses 24 hours apart*

Substance	Dose ($\mu\text{g.}$)	No. of mice	% +ve response	Slope \pm S.E.*
International Standard Oestrone	0.0002	20	15	
	0.00025	20	25	
	0.0003	20	70	5.441 ± 0.638 (excluding last 3 doses)
	0.0004	20	85	
	0.0005	80	87.5	
	0.00075	20	90	
	0.001	20	100	
	0.0015	20	95	
Oestradiol	0.00025	40	37.5	
	0.0005	40	47.5	0.850 ± 0.440
Oestriol	0.00025	20	0	
	0.0005	40	2.5	8.726 ± 1.209
	0.001	20	55	
Diethylstilboestrol	0.00025	40	32.5	
	0.0005	40	70	3.249 ± 0.474

*Standard error.

of interest are that the dose/response curves are as steep as those found when the substances are given subcutaneously, with the exception of oestradiol, and that the difference between any two slopes exceeds in all cases twice its standard error. This unfortunate discrepancy makes it improbable that the intravaginal route is adaptable for assaying oestrogenic preparations accurately, unless their exact nature is known.

The fairly detailed dose/response curve established for oestrone has the same slope as that found with similar mice when the hormone is injected in oil [Emmens, 1939b] if the three highest doses are excluded. These, which from the remaining responses would be expected to have produced 100% of positive responses, tend to fall below that level. It has been a general experience with the intravaginal method that responses of more than 90% are difficult to obtain unless relatively large doses are used, as

one or two mice in a group often fail to react at the higher dose-levels—due, in all probability, to leakage of the solution from the vagina. It is therefore essential not to rely upon high response levels for assaying preparations. When suspensions of the less active synthetic compounds are used, it is also occasionally found that an unexpected positive response occurs at a low level of dosage. This again may be explained on the assumption that a relatively large particle of the substance has entered the vagina despite the precautions taken, and a repetition of the test at the same and higher levels clarifies the situation. When dealing with such suspensions, it has been the practice never to rely upon the results of a single assay, but to repeat all tests at two dose-levels at least, and to establish further dose-levels at which definitely negative responses are obtained.

In Table II are shown the results with the oestrogens of Table I when compared by a single intravaginal application. The three most potent

Table II. *Response of ovariectomized mice to oestrogens given by a single intravaginal application in 50% water-glycerol*

Substance	Dose (μ g.)	No. of mice	% +ve response
International Standard	0.0015 0.003	40 20	25 60
Oestrone			
Oestradiol	0.0015	20	60
Diethylstilboestrol	0.0015	20	65
Oestriol	0.01	20	0

compounds, oestrone, oestradiol and diethylstilboestrol remain of approximately equal activity. Oestriol had about a third of the activity of the others when compared by giving two doses, but now appears to be less potent in comparison with them. The figures are explicable on the same general grounds as those found when these oestrogens are compared by giving a single subcutaneous injection, in that the individual rates of absorption and elimination are able to exert a greater effect on the apparent potency than when multiple injections are given. It seems probable that two injections in 50% glycerol usually enable something approaching complete utilization of the material to be effected, and this is reflected in the striking levelling of potencies seen with the four compounds.

The subcutaneous/intravaginal ratios

The ratio of the median effective subcutaneous dose (that required to give 50% of positive responses) to the corresponding intravaginal dose is

the S/L (systemic:local) ratio. The S/L ratio for the three natural oestrogens at the top of Table III shows considerable variation, but the

Table III. *Substances having a high S/L ratio*

No.	Substance	Median effective dose when given by:		S/L ratio
		Subcut. injection	Intravaginal application	
1	Oestrone	0.075 µg.	0.00029 µg.	260
2	Oestrone methyl ether	0.9 "	0.015 "	60
3	Oestradiol	0.025 "	0.0005 "	50
4	Ethinyl oestradiol	0.03 "	0.00025 "	120
5	Oestriol	2.0 "	0.001 "	2000
6	Ethinyl-dihydro-equilin	0.045 "	0.0005 "	90
7	Diethylstilboestrol	0.12 "	0.00037 "	320
8	ψ-Diethylstilboestrol	0.45 "	0.001 "	450
9	Diethylstilboestrol dimethyl ether	8.0 "	0.02 "	400
10	Ethyl-propyl-stilboestrol	0.6 "	0.0035 "	170
11	Di-iso-propyl-stilboestrol	4.7 "	0.015 "	310
12	Di-n-butyl-stilboestrol	50.0 "	0.16 "	310
13	Hexoestrol (Meso)	0.16 "	0.0009 "	180
14	Hexoestrol (Racemic)	8.9 "	0.025 "	360
15	4:4-Dihydroxy-γ:δ-diphenyl-β:δ-hexadiene	0.1 "	0.00058 "	170
16	Triphenylchloroethylene	65.0 "	1.0 "	65
17	3:3':4:4'-Tetrahydroxy-γ:δ-diphenyl-n-hexane	12.5 "	0.2 "	63
18	1-Ethyl-2-(p-hydroxyphenyl)-6-hydroxy-1:2:3:4-tetrahydronaphthalene	ca. 1.0 mg.	ca. 8.0 "	ca. 125

high figure of 2000 found with oestriol is particularly exceptional, as it so happens that oestriol, presumably on account of its great solubility in body-fluids, is the only substance investigated which has been found to increase greatly in potency when more than two subcutaneous injections are given. With four injections the median effective dose is 0.14 µg., which gives a ratio of 140. The ratio for the other naturally occurring oestrogens would be very little affected by an increase in the number of injections [cf. Emmens, 1939a], as they do not show much change in potency.

The expectation discussed above, that oestrogens would fall into two distinct classes, has been amply confirmed by these tests. The substances in Table III, the true oestrogens, in no case exhibit a ratio of less than 50, whereas those in Table IV, the pro-oestrogens, in no case exhibit a ratio of more than 2, when this ratio has been fully investigated. It has proved impossible to determine all of the ratios listed in Table IV completely, sometimes because of lack of material, as it takes a solution or suspension at 100 mg./ml. to give a dose of 2 mg., and sometimes because higher concentrations would not pass through the needle of the syringe.

As it is usually easier to determine the high S/L ratios with fair

Table IV. *Substances having a low S/L ratio (pro-oestrogens)*

No.	Substance	Median effective dose when given by:		S/L ratio
		Subcut. injection	Intravaginal application	
19	4-Hydroxydiphenyl	120 mg.	> 10 mg.	< 12.0
20	4: 4'-Dihydroxydiphenyl	15 "	> 2 "	< 7.0
21	4-Hydroxydiphenyl ether	30 "	> 4 "	< 7.0
22	4: 4'-Dihydroxydiphenyl ether	30 "	> 2 "	< 15.0
23	Stilbene	3.2 "	2 "	1.6
24	4-Hydroxystilbene	2 "	> 2 "	< 1.0
25	4: 4'-Dihydroxystilbene	2.2 "	> 2 "	< 1.0
26	4-Hydroxy- α : β -diethylstilbene	20 μ g.	> 30 μ g.	< 0.6
27	α -Phenyl-stilboestrol	15 "	10 "	1.5
28	α -Phenyl- β : β -di(<i>p</i> -hydroxyphenyl)-ethylene	8 "	20 "	0.4
29	Dihydroxyhoxahydrochrysono	150 "	ca. 200 "	ca. 0.7
30	9: 10-Dihydroxy-9: 10-di- <i>n</i> -propyl-9: 10-dihydro-1: 2: 5: 6-dibenzanthraceno	18 "	20 "	0.9
31	9: 10-Dihydroxy-9: 10-di-cyclopentyl-9: 10-dihydro-1: 2: 5: 6-dibenzanthraceno	490 "	300 "	1.6
32	Dihydroxy-di- α -naphthyl-acenaphthene	200 "	ca. 600 "	ca. 0.33
33	<i>p</i> -Hydroxypropionophenone	8 mg.	ca. 2 mg.	ca. 4.0
34	<i>p</i> -Hydroxypropionophenone pinacol	30 μ g.	60 μ g.	0.5
35	<i>p</i> - <i>tert</i> -Amyl-phenol	40 mg.	> 20 mg.	< 2.0
36	4-Hydroxy-triphenylmethane	6 "	> 2 "	< 3.0
37	<i>trans</i> -Androstenodiol	0.7 "	0.8 mg.	0.9
38	Anhydro-hydroxy-progesterone	1.2 "	1.0 "	1.2
39	α -Ethylstilboestrol	ca. 90 μ g.	> 20 μ g.	< 4.0

accuracy, the degree of variation shown in Table III is probably indicative of real but small differences between some of the compounds. On the other hand, none of the figures for the low S/L ratios can be considered to differ significantly from unity.

In Table V, some esters of substances having a high S/L ratio are compared with the parent compounds. It will be seen that esterification, which may raise the S/L ratio, does so mainly by virtue of its effect in increasing the dose needed by subcutaneous injection. Only small differences were found, on a molar basis, between the amounts of the esters and parent compounds needed to cause cornification when given intravaginally. Such esters may therefore be supposed to be hydrolysed locally with high efficiency. The effective intravaginal doses of the lower esters of oestrone and of oestradiol benzoate are somewhat less, in terms of free hormone, than those of the unesterified substances. It seems probable that these esters are rather more efficient than the free hormones, on account of a slight decrease in the rate at which the active material becomes available, and a corresponding decrease in the rate of its absorption and further metabolism.

Intraperitoneal injection

Pincus & Werthessen [1938] report that some synthetic compounds, notably phenanthrene derivatives, are very much more potent when given intraperitoneally than they are when injected subcutaneously. Increases in potency were found of the order of 50–300 times. They suggest that absorption is facilitated when these substances are injected into the body cavity.

Table V. *The effect of esterification on the S/L ratio*

Substance	Median effective dose by subcut. injection	Median effective dose by intravaginal application:		S/L ratio (actual)
		Actual	As free substance	
Oestrone	0.075 µg.	0.00029 µg.	0.00029 µg.	260
Oestrone butyrate	0.070 "	0.0003 "	0.00022 "	230
" caproate	0.15 "	0.0003 "	0.00021 "	500
Oestradiol	0.025 "	0.0005 "	0.0005 "	50
Oestradiol benzoate	0.08 "	0.0005 "	0.00034 "	160
Diethylstilboestrol	0.12 "	0.00037 "	0.00037 "	320
Diethylstilboestrol dipropionate	0.14 "	0.00075 "	0.00045 "	190
Diethylstilboestrol dicaproate	0.45 "	0.0015 "	0.00075 "	300
Diethylstilboestrol dipalmitate	6.0 "	0.002 "	0.00062 "	3000

Compounds 18, 20, 23, 24, 25, 27 and 35, a series containing some members of low activity, which might be supposed to be more effective intraperitoneally, were therefore given to groups of spayed mice by two intraperitoneal injections in oil, in one tenth of the dose needed to give 50% of positive responses by subcutaneous administration. The assays were carried out as usual, except that smears were taken on the day of the 2nd injection and for two further days. No positive responses were observed.

It does not appear, therefore, that the use of intraperitoneal injections would have given very different ratios from those found by the present method. The absorption of the compounds tested from the subcutaneous tissues and from the vagina does not appear to have been as inefficient as Pincus & Werthessen's results—admittedly with different materials—might have led one to suspect.

DISCUSSION

The structural formulae of the substances showing a high S/L ratio are shown in Fig. 1, with index numbers corresponding to those in Table III. This class includes the natural oestrogens and various modifications of them, and their esters, the synthetic oestrogens of the stilboestrol series, hexoestrol and dihydroxydiphenylhexadiene, excluding, however, dihydroxystilbene and monoethylstilboestrol. It also includes compound

18, and, rather unexpectedly, triphenylchloroethylene. With the exception of this last substance, the high-ratio compounds are thus related stereochemically, although the *cis*-derivative, ψ -diethylstilboestrol [Dodds, Golberg, Lawson & Robinson, 1939] and the externally compensated form of hexoestrol (*rac.*-hexoestrol —m.p. = 128°) are included. Compound 17 and triphenylchloroethylene seem to have very flat intravaginal dose/response curves, but there remains little doubt that they are to be classed with the high-ratio compounds, as they have consistently given positive responses with doses of the order indicated in Table III, although high percentage responses are difficult to obtain.

The formulae of substances with a low S/L ratio are shown in Fig. 2. The importance of small details of structure is shown by the finding that 4-hydroxy- α : β -diethylstilbene, α -ethylstilboestrol (mono-ethylstilboestrol), *p*-hydroxypropiophenone pinaeol (4:4'- α : β -tetrahydroxy-diethyldibenzyl) and dihydroxyhexahydrochrysene fall into this class, as they are all closely related to members of the high-ratio series. Dihydroxyhexahydrochrysene, said by Dodds *et al.* [1939] to be the *trans*-compound, might in particular have been expected to possess a high S/L ratio, as it so closely resembles the natural oestrogens, and in fact possesses the structure which diethylstilboestrol has been supposed to imitate in the disposition of its ethyl groups (Fig. 3).

It is evident that a considerable range of compounds will have to be investigated before the structural configuration which must be possessed by a true oestrogen becomes clear. The high intravaginal activity of triphenylchloroethylene disposes of any supposition that hydroxyl groups and a close resemblance to the natural compounds is essential, unless we further suppose that it is converted locally to a different compound. Fuller discussion of this question seems best avoided until more data are available, but it may be noted that diethylstilboestrol is no longer a direct oestrogen if a hydroxyl group or an ethyl group is missing from the molecule.

The pro-oestrogens listed in Table IV and Fig. 2 may with some degree of confidence be described as compounds which must undergo structural changes in the body before becoming oestrogens. It need not be emphasized that a rigid proof of this doctrine is not presented here, but that it is so far a successful working hypothesis, and that it is not necessary to suppose that more than a minute fraction of such compounds is so transformed. There appear to be no grounds for supposing that they stimulate the production of a natural oestrogen by the animal. Such oestrogen production in spayed animals has not been conclusively demonstrated, and the chemical structure of at least some of these compounds is such that the small changes necessary for their conversion to oestrogens seem

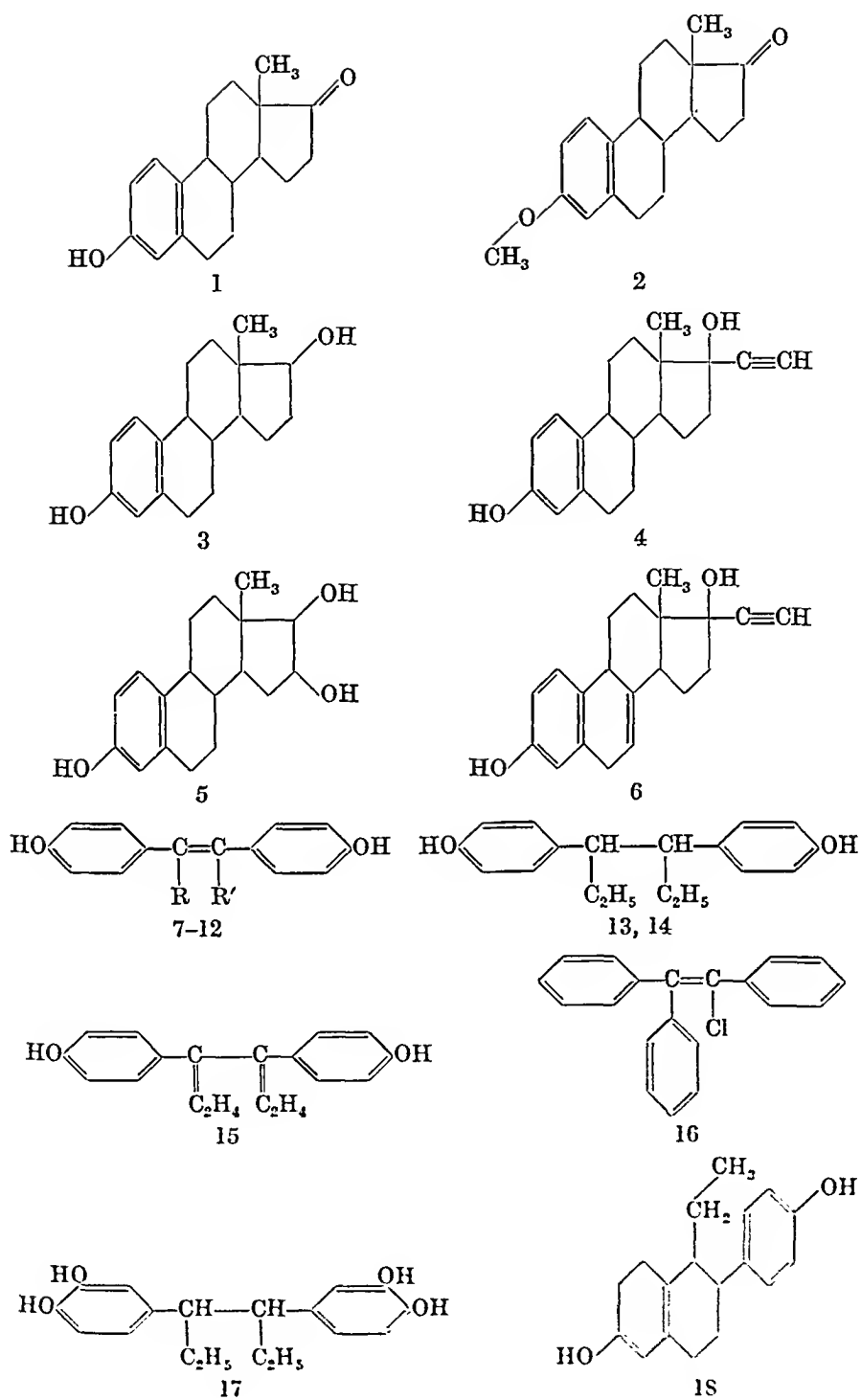


Fig. 1. Oestrogens (Table III)

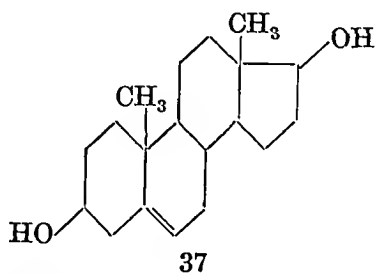
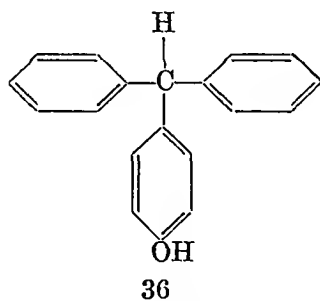
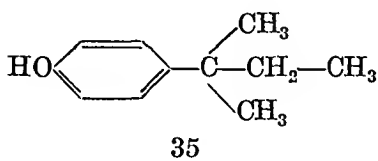
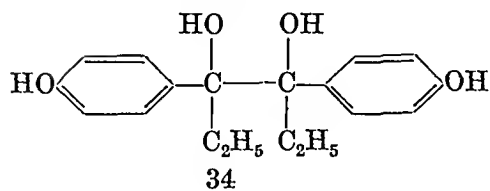
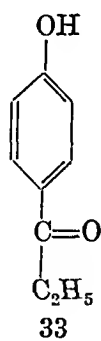
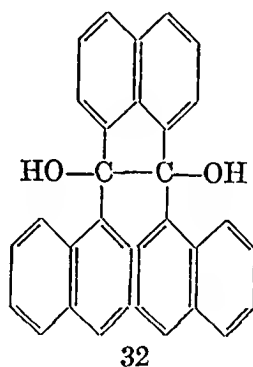
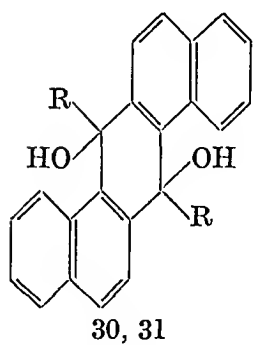
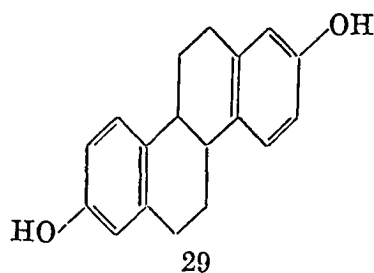
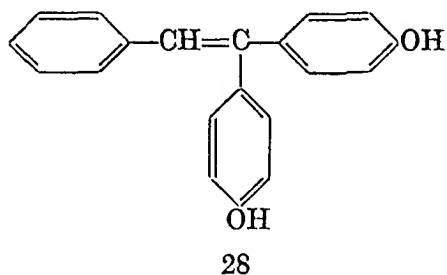
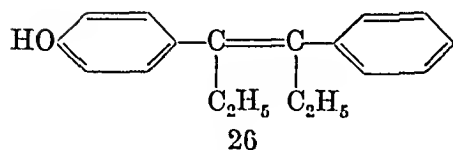
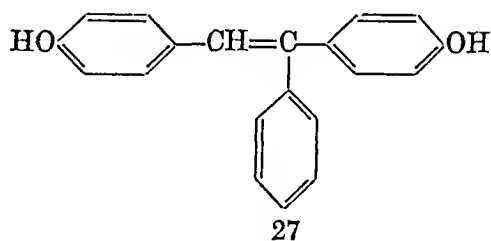
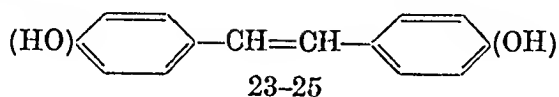
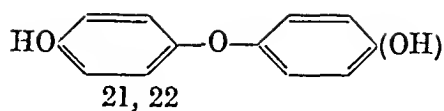
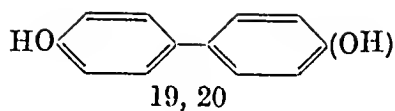


FIG. 2. Continued Opposite

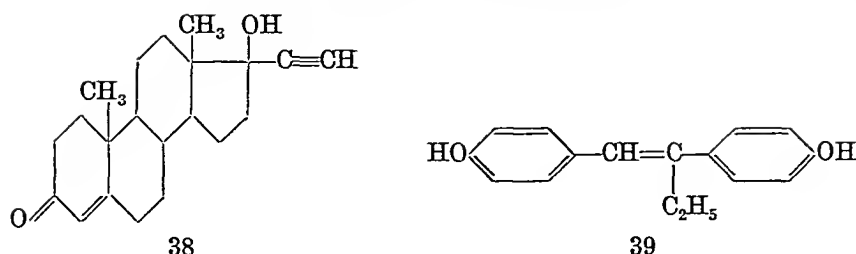


FIG. 2. Pro-oestrogens (Table IV)

more likely to take place in the body than a secretion of abnormally high amounts of oestrogen from extra-ovarian sources, under stimulation from the injected substance.

The oestrogens listed in Table III, on the other hand, are possibly active in their initial form, and very probably so in the case of the natural free oestrogens. Local changes very likely take place in the case of the methyl ethers, and possibly with the ethinyl compounds. The esters are almost certainly hydrolysed. The methyl ethers are not as active intravaginally as are the parent substances, and we may suppose that demethylation takes place before they act, but that it is not rapid or is not complete.

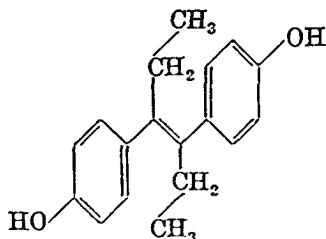


FIG. 3. Diethylstilboestrol (*trans*-configuration)

The peculiarities of the segregation into the classes of low and high S/L ratio discussed above may lead to suspicions that classification into pro-oestrogens and oestrogens on this basis alone is not sufficiently justified, and that the ratio may in some cases depend on properties of another kind. The outstanding probability is related to the rate at which compounds may be absorbed into the general circulation. Thus, if a substance were rapidly and completely absorbed by the vaginal mucosa it might become so quickly distributed throughout the system as to produce no more effect than if it were injected elsewhere, not remaining long enough at a high concentration in the vagina. This might cause a directly active substance to appear relatively inactive when given locally, and to possess a low S/L ratio. Moreover, such rapid absorption must be postulated in order to explain the metabolism of pro-oestrogens in the body, as these

substances or their metabolites stimulate cornification when given in effective doses *per vaginam* as rapidly, or nearly as rapidly, as the natural hormones. The natural hormones and the highly active synthetic compounds are not dispersed throughout the body, since they are active in a small fraction of the dose required when such dispersal occurs, as by subcutaneous injection. If all the compounds examined were, for instance, true oestrogens, and underwent varying degrees of absorption, they should show a wide, continuous range of S/L ratios. The fact that they do not, but segregate into two distinct classes is a strong argument in favour of the hypothesis put forward.

Robson & Adler [1940] have also shown that oestradiol and stilboestrol act locally on the vagina without appreciable absorption into the general circulation, since a separate vaginal pocket formed from the lower vagina was practically unaffected in mice receiving the compounds in saline in the upper vagina. Their results are particularly striking, since as much as 0.2 μ g. of diethylstilboestrol given into one part of the vagina produced almost no response in the second, separated part. They also remarked that oestriol glucuronide is active locally.

The present results are thus explicable on the grounds that physiological doses of substances which are active locally are fixed or utilized in the tissues, and do not escape in appreciable amounts into the circulation, whereas the pro-oestrogens are not immediately utilized or fixed, but pass into the circulation and undergo metabolic changes to oestrogens.

The two compounds possessing other activities than those typical of oestrogens are of interest. *trans*-Androstenediol is also and more predominantly an androgen, and anhydro-hydroxy-progesterone (ethinyl testosterone) is also androgenic, progestational and metrotrophic [Emmens & Parkes, 1939b]. Emmens & Parkes [1939a] have already suggested that such compounds, which exhibit more than one type of biological activity, do so by virtue not of the multiple properties of a single molecule, but of the separate properties of various metabolic products. This has now been demonstrated as regards the oestrogenic activity of these two substances. On the other hand, *trans*-androstenediol (unpublished data) and anhydro-hydroxy-progesterone [Emmens & Parkes, 1939b] are highly active as androgens when injected to the capon comb, so that the intact molecules are apparently androgenic.

As observed by Mühlbock [1940], the potencies of oestrone, oestradiol and diethylstilboestrol are not significantly different when these oestrogens are given intravaginally. It is difficult to say whether any great importance is to be attached to this, but the virtual equality of the three most highly active substances seems to suggest that they are as fully efficient as an oestrogen can be. The same situation is found with the androgens

[Dessau, 1937; Emmens, 1938, 1939a and further unpublished data], in that androsterone, testosterone and androstenedione differ very little in potency when injected into the capon comb, and *trans*-androstenediol, androstenediol and *trans*-dehydro-androsterone are only slightly less potent, showing in all a range of from 10 I.U. to 3 I.U. per mg., whereas, by injection, the range for these six androgens is from 70 I.U. to 2 I.U. per mg. No 'pro-androgen' has yet been discovered.

It should be noted that, as far as the evidence goes, oestrogens do not act directly on the epithelial cells of the vagina [Emmens & Ludford, 1940], their action being possibly confined to the production of local hyperaemia [Hechter, Lev & Soskin, 1940]. This is in accord with the finding of a high local potency for substances which are presumed to act unchanged, or to undergo metabolic changes which can be effected in the vagina itself.

SUMMARY

1. The natural oestrogens, many compounds of the stilboestrol series, hexoestrol, and a few other synthetic oestrogens are highly active when administered intravaginally in 50% aqueous glycerol. The ratio of the dose which produces 50% of cornified smears when given subcutaneously in oil, to that required intravaginally (the systemic/local or S/L ratio) is greater than 50, and usually falls between 50 and 400.

2. Other synthetic compounds do not produce vaginal cornification unless they are given intravaginally in about the same quantity as is needed by injection. The S/L ratio for these substances therefore approximates to unity. It is suggested that these compounds, the 'pro-oestrogens', are absorbed into the circulation and undergo metabolic changes to true oestrogens. *trans*-Androstenediol and anhydro-hydroxy-progesterone also fall into this class.

3. Esters of oestrone, oestradiol and diethylstilboestrol approximate in local activity to the free substances, and are most probably hydrolysed locally with high efficiency.

4. Further investigations are planned by means of which it is hoped to elucidate the course of metabolism of the pro-oestrogens and the structure which must be possessed by substances of the high S/L ratio group.

I am deeply indebted to Professor E. C. Dodds and Mr. W. Lawson who have generously provided the great majority of the synthetic compounds used in this investigation. I have also to thank Dr. H. King for hexoestrol and *rac*-hexoestrol. Dr. P. G. Marshall and the British Drug Houses, Ltd., for the di-methyl ether and di-esters of stilboestrol, and

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THE ANTERIOR PITUITARY GLAND AND PROTEIN METABOLISM

I. THE NITROGEN-RETAINING ACTION OF ANTERIOR LOBE EXTRACTS

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It has been realized for some years that treatment with anterior pituitary extract may be followed, under some conditions, by a positive nitrogen balance [Teel & Cushing, 1930; Gaebler, 1933; Lee & Schaffer, 1934] and a fall in the non-protein nitrogen content of the blood [Teel & Watkins, 1929]. As might be expected such effects are associated with growth-promoting fractions of the anterior pituitary lobe.

Evidence is now accumulating which relates the influence of anterior lobe preparations on growth and nitrogen retention with their action on carbohydrate metabolism. Thus Shipley & Long [1938] and Young [1939a] found that anterior pituitary fractions which possess diabetogenic activity are also growth-promoting, while more recently Dohan, Fish & Lukens [1941] found that dogs which are in the process of being made permanently diabetic as the result of treatment with crude anterior pituitary extract, exhibit nitrogen retention during those periods in which there was little glycosuria attributable to protein sources. Mirsky [1939] suggested that the anterior pituitary lobe exerts two types of action on protein metabolism in the normal animal, one being a direct stimulation of protein catabolism in the muscles, while the other is an indirect action through the pancreas, leading to the anabolism of protein material. The pancretotropic action of anterior pituitary preparations, which results in an increase in the amount of pancreatic-islet tissue and in the amount of insulin extractable from the pancreas [Richardson & Young, 1937; Marks & Young, 1940] is in harmony with such an idea, and Young [1939b, 1940] considered the possibility that the diabetogenic, pancretotropic, nitrogen-retaining and growth-promoting activities of such preparations are all due to the same substance. Such an idea may be an over-simplification of a complex problem [cf. Gaebler & Galbraith, 1941] but nevertheless it is clear that the effects of anterior lobe extracts on the metabolism of protein and on that of carbohydrate may be intimately related.

Cuthbertson, McCutcheon & Munro [1940] found that when the carbo-

hydrate and protein moieties of the diet of the rat are given at different times during the day, protein catabolism is somewhat greater than during a period in which the same constituents of the diet are offered simultaneously. The present paper is concerned with the influence of suitable crude pituitary extracts, known to have nitrogen-retaining activity in the rat, on this phenomenon.

METHODS

Diet

In the first series of experiments, designed to determine the most suitable type of crude pituitary extract active in producing nitrogen retention in rats ingesting a constant amount of food each day just sufficient to maintain body-weight, the animals were fed on 'Purina Fox Cubes'¹ and water, which constitutes a complete diet. The rats were kept in metabolism cages for 22 hours each day during which urine (and in some instances, faeces) was collected. For two separate hours, one in the morning and the other in the evening, the rats were moved into ordinary cages together with their ration of food for the day, this being rapidly and completely consumed in most cases. The observed figures for urinary nitrogen excretion were multiplied by the factor $24/22 = 1.091$ in order to obtain values applicable to the whole period of 24 hours.

In subsequent experiments in which the carbohydrate and protein moieties of the diet were fed separately, the diet and procedure were those described by Cuthbertson *et al.* [1940].

Animals and procedure

Male Wistar-strain albino rats were used in these experiments, usually in groups of five, the results for which were averaged.

In the experiments in which a mixed diet was given observations of nitrogen output were made during a preliminary control period of 2-5 (usually 2) days; pituitary extract was then administered daily for a similar period and then followed a second control period. In some experiments an extract of thymus gland was injected daily during the control

¹ Manufactured by the Ralston Purina Company, Woodstock, Ontario, Canada. According to the manufacturers the ingredients are: flavin concentrate, carotene, wheat germ, dried skim milk, liver meal, dried brewer's yeast, barley malt, fish meal, dried meat, alfalfa meal, corn grits, soya bean oil meal, molasses, dried beet pulp, cod liver oil, 1% steamed bone meal, 1% iodized salt.

The manufacturer's guaranteed analysis is:

Crude protein, not less than 20%.

Crude fat, not less than 3%.

Crude fibre, not more than 6%.

Our own analyses showed approximately 20% protein on one occasion, and 20.4% on another.

periods in order to reveal any non-specific effect of inert protein material, while in others daily injections of saline were given during the middle or experimental period instead of pituitary extract. No influence of the thymus extract on nitrogen retention was observed and the results for control periods in which injections of thymus extract were given have accordingly not been considered separately from other control periods.

In those experiments in which the carbohydrate and protein fractions of the diet were given at different times the various periods of observation—control and experimental—lasted 4–7 days, and again in some instances thymus extract was injected daily during a control period.

All injections were given by the subcutaneous route.

Estimations

The urine collected daily from each group of rats was pooled for analysis. Total nitrogen was determined by Kjeldahl's method, and creatine and creatinine by the method of Folin [1914]. Occasional estimations of the total nitrogen content of the faeces were made by Kjeldahl's method.

Anterior pituitary extracts

The pituitary glands used in the present investigation were obtained from the slaughterhouse in an absolutely fresh condition and brought to the laboratory frozen in solid carbon dioxide. In one experiment, however, a sample of commercial acetone desiccated ox anterior lobe powder was used.

The fresh pituitary glands were dissected while in a frozen condition, a clean separation being effected between the tissue of the anterior lobe and that of the posterior part. The anterior lobes were then minced while in a frozen condition and then either desiccated in acetone or alcohol (the desiccated material being subsequently used for the preparation of an alkaline aqueous extract), or extracted immediately with alkaline saline in the cold. In all instances the final concentration of the extracts was so adjusted that 1 ml. contained the material extracted from the equivalent of 50 mg. of dried gland, the assumption being made if necessary that 1 g. of fresh anterior lobe yielded 200 mg. of desiccated tissue.

(a) *Crude alkaline extract* was prepared by grinding the glands with saline at pH 8.5 in the cold store, as previously described [Young, 1938]. The extract of thymus gland for control experiments was prepared in a similar manner from frozen fresh calf thymus tissue.

(b) *Extract of acetone-dried glands.* The fresh minced anterior lobe tissue was stirred at intervals with twenty volumes of cold absolute acetone in the cold overnight. The residue was again extracted with acetone and finally washed with alcohol and ether and dried *in vacuo* over calcium chloride. The dried tissue was extracted twice with saline at pH 8.5 [Young, 1938].

(c) *Extract of alcohol-dried glands.* The fresh minced anterior lobe tissue was dried either with absolute alcohol, with commercial methylated spirit ('Methcol') or with absolute alcohol containing 5% methyl alcohol. The procedure was essentially that of Collip [1939], according to which the minced tissue was first stirred with four volumes of the solvent and the residue re-extracted twice with a similar volume. The tissue dried thus was subsequently extracted with saline at pH 8.5.

RESULTS

Animals receiving a mixed diet

The results for experiments in which a crude alkaline extract of fresh pituitary tissue was used, are summarized in Table I, in which the data for different experiments each carried out on groups of 5 rats, are averaged. From this it is seen that the daily injection of an extract corresponding to 10 mg. of dried ox pituitary tissue gives a maximal effect, resulting in a retention of approximately 20% of the initial amount of nitrogen (about 200 mg./rat/day) excreted in the urine. Sheep pituitary tissue appears to be at least as active as ox pituitary tissue in this respect (Table I). In all instances the retention of nitrogen was accompanied by an increased rate of growth (Table I). During the post-injection control period, the nitrogen excretion consistently rose to a level substantially above that during the pre-injection control period, but this loss of nitrogen was not, in general, accompanied by a fall in body-weight.

Table II summarizes the data relating to extracts of pituitary tissue

Table II. *Influence of extracts of dried anterior lobe tissue of ox pituitary gland on nitrogen excretion of 'purina-fed' rats*

Method of desiccating pituitary tissue	Dose injected daily (Mg.-equiv. dried tissue)	Rats		Average daily food consumption (g.)	Average urinary nitrogen excretion (mg./rat/day)		
		Total number in group	Average initial weight (g.)		Pre-injection period	Injection period	Decrease during injection period (%)
Absolute acetone	100	5	127	10.0	235	188	20.0
	50	15	162	10.6	265	213	19.6
	10	5	134	12.0	276	225	18.5
	1	5	135	12.0	308	285	7.5
Absolute alcohol	50	10	163	10.6	273	264	3.3
'Methcol'	100	10	205	11.4	328	310	5.6
	50	10	165	10.6	281	262	6.8
Absolute alcohol containing 5% methyl alcohol	50	10	164	11.0	294	283	3.9

Table I. *Influence of crude alkaline extract of anterior pituitary tissue on the nitrogen excretion of the 'purina'-fed rat*

Source of pituitary extract	Dose injected daily (Mg.-equivalent of dried tissue)	Total number in group	Average initial weight (g.)	Average (urinary) nitrogen excretion			During injection period		
				Average daily food consumption (g.)	Pro-injection period	Injection period (mg./rat/day)	Post-injection period	Increase in rate of growth (g./day)	Decrease in urinary nitrogen excretion (%)
Ox glands	100	10	122	10.8	187	145	220	1.8	22.4
	50	10	133	10.0	190	145	199	2.2	23.7
	10	20	142	10.6	213	166	240	1.5	22.0
	5	10	114	9.4	171	158	—	1.2	8.2
	1	10	121	10.8	173	170	207	0.5	1.7
Sheep glands	50	5	117	10.0	198	157	213	1.7	20.7
	5	5	112	10.0	190	156	207	1.6	17.9
	0.5	5	106	10.0	180	172	205	0.8	4.4
Control (saline 1-2 ml.)	—	15	108	10.0	270	264	—	0.6	2.2